Cytomegalovirus Promoter Up-regulation Is the Major Cause of Increased Protein Levels of Unstable Reporter Proteins after Treatment of Living Cells with Proteasome Inhibitors*

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Fluorescent unstable proteins obtained by the fusion of a fluorescent protein coding sequence to the N-terminal threonine of three of the β-subunits (β1, β2, and β5) of ubiquitin/proteasome system (UPS) consol-trans-acting factor into the catalytic chamber of the protein translocation into the catalytic chamber of the proteasome (PDI) mRNA in samples 2, 5, and 6, respectively.

This article has been withdrawn by the authors. In Fig. 3, the same images were used to represent the results of different experimental conditions for EGFPd2 mRNA levels between samples 6 and 7 and samples 12 and 13 and for β-actin mRNA between samples 1 and 2. The background was inappropriately adjusted in the tubulin panel. In Fig. 7, the same images were used to represent different experimental conditions for protein-disulfide isomerase (PDI) mRNA in samples 2, 5, 6, and 9, and 11 and 12. Additionally, sample 3 was re-used as sample 4 and for ribosomal RNA (rRNA). In Fig. 8c, the same image was used to represent the results of different experimental conditions between GFPu (lane 9) and EYFP (lane 9). In supplemental Fig. 2, the same images were used to represent the results of different experimental conditions for PDI mRNA samples 5 and 6. In supplemental Fig. 6, the same images were used to represent the results of different experimental conditions for β-actin mRNA between samples 2 and 4 and between samples 6 and 7. The same images were used to represent the results of different experimental conditions in the tubulin immunoblots between sample 2 in the left hand panel and sample 1 in the right hand panel.

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†The abbreviations used are: UPS, ubiquitin/proteasome system; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus; CREB, CAMP-responsive element-binding protein; RT, reverse transcription; qPCR, quantitative real time PCR; ActD, actinomycin D; CHX, cycloheximide; EYFP-C-Cot, EYFP-C-terminal Cot; C7, threshold cycle.

The ubiquitin/proteasome system, in the degradation of many proteins in eukaryotes, constitutes a basic cellular machin-ery that seems to freely diffuse into the catalytic chamber of the proteasome. The 20S proteasome is a multicatalytic protease; its structure can be described as a heterodimeric cylinder composed of two heptameric outer α-rings and two inner β-rings. The proteolytic activity of the proteasome is due to the multiple isopeptide linkages between a lysine residue of the protein substrate. This is usually transferred to a member of the E3 ubiquitin ligase family by ubiquitin/proteasome system (UPS). The ubiquitylated protein is then covalently linked to the protein substrate. This is responsible for the covalent linkage of ubiquitin by E3 enzymes (3). These modular sequences, or degrons, can often be transferred in-frame to otherwise stable proteins and promote the degradation of the fusion protein by the ubiquitin/26S proteasome system.

In recent years several groups have developed protein constructs for measuring UPS activity in cells and animals. These constructs can be easily detected by direct fluorescence, chemiluminescence, or color development. Examples of these reporter proteins are green fluorescent protein and their derivatives, luciferase, lactamase, and β-galactosidase (4). Each of these reporter proteins is stable proteins, and in-frame ligations with specific degrons from unstable proteins produces shortening of the half-life of the fusion proteins. One type of such constructs is based on the removal of ubiquitin from ubiquitin N-terminal fusion proteins rendering different N-terminal residues (4) or linking a modified ubiquitin (G76V) in the N-terminal amino acid) is responsible of the covalent linkage of ubiquitin by E3 enzymes (3). These modular sequences, or degrons, can often be transferred in-frame to otherwise stable proteins and promote the degradation of the fusion protein by the ubiquitin/26S proteasome system.

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terminal of the green fluorescent proteins (5) or luciferase (6) creating a signal for the ubiquitin fusion degradation pathway. In these cases the resulting proteins, either after ubiquitin removal and N-terminal recognition or directly by the ubiquitin fusion degradation pathway, are degraded after ubiquitlation by the 26 S proteasome (3, 4). More general UPS substrates have been designed by the fusion of the GFP to a degron found in unrelated proteins. Two main degrons have been used for fusion to the C-terminal end of GFP, the CI1 degron (ACKNWFFSLHVFVIHL) found in a yeast screening for sequences targeting to the endoplasmic reticulum degradation pathway (7) and the C-terminal (amino acids 422–461) region of ornithine decarboxylase (8). Although GFP-CI1, also called GFPu, likely requires ubiquitilation prior to its degradation (9), the second type, commercially named EGFPd2 (BD Biosciences), seems to be directly degraded by 26 S proteasome without the requirement of prior ubiquitilation (8, 10). Other specific substrates have been generated, such as IκBα–GFP (11) and IκBα–luciferase (6), based on the short half-life of IκBα, which is unstable due to the targeting of IκBα by stress or cytokines to degradation by the UPS.

A general property of all these constructs in cells or in transgenic animals is the low steady-state level of expression of the reporter fusion protein attributed to the short half-life of the reporter protein and its rapid decay to almost undetectable levels when protein synthesis is inhibited because of its rapid degradation by the UPS. In agreement with the above conclusion, treatment of the transfected cells (or animals) with proteasome inhibitors produces a variable 2–10-fold increase in the amount of the unstable reporter protein (see references cited above). We have recently identified a putative C-terminal degron in the study of a very significant increase in the amounts of the unstable reporter fusion protein attributed to the short half-life of the reporter protein and its rapid decay to almost undetectable levels when protein synthesis is inhibited because of its rapid degradation by the UPS. In agreement with the above conclusion, treatment of the transfected cells (or animals) with proteasome inhibitors produces a variable 2–10-fold increase in the amount of the unstable reporter protein (see references cited above). We have recently identified a putative C-terminal degron in the study of the degradation of Cot/Tpl2 kinase (12). Therefore, we decided to characterize EYFP-C-Cot (amino acids 390–435) as another reporter that can be used in living cells to gauge UPS activity. In the course of our studies we found a situation similar to that described above, low levels of expression in transfected cells and a very significant increase in the amount of the unstable protein in cells treated with different proteasome inhibitors. Further experiments demonstrated that the increase in reporter protein abundance in the cells after treatment with proteasome inhibitors is mainly due to an increase of the transcription of the reporter gene due to up-regulation of the CMV promoter by proteasome inhibitors, and the accumulation of the unstable fusion protein can be completely prevented by co-treatment with transcriptional inhibitors. We have generalized these results with other unstable fusion proteins and actually found that the up-regulation of the CMV promoter by proteasome inhibitors, mainly by API transcription factor, and not inhibition of the degradation of the reporter protein is the main cause of the accumulation of the unstable reporter protein in the cell.

EXPERIMENTAL PROCEDURES

Cell Lines, DNA Constructs, and Transfections—Rat pheochromocytoma PC12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% horse serum and 5% fetal bovine serum. NIH3T3 (mouse), N2A (mouse), and HeLa (human) cells were cultured in the same medium containing 10% fetal bovine serum. The reporter fluorescent constructs used in this study are EYFP-Cot/Tpl2, which contains the EYFP coding sequence fused with the C terminus of Cot/Tpl2 (amino acids 390–435) (12); EGFPd2, which contains the EGFP coding sequence fused with the C-terminal sequence of ornithine decarboxylase (amino acids 422–461) obtained from BD Biosciences; and GFP-CI1, which contains the GFP coding sequence followed by the CI1 sequence (ACKNWFFSLHVFVIHL) that was obtained from ATCC (8–10). All the above constructs as well as the corresponding EYFP and EGFP vectors (Clontech) used as controls are under the transcriptional control of the CMV promoter. Firefly luciferase transcriptional reporters for AP1 (three AP1 binding sites) (13), CREB (three CREB binding sites) (14), and NFκB (three κB sites) (15) used in competition experiments have been described previously. The empty vectors pcDNA3.1 (Invitrogen) and pEF-BOS (16) were used as DNA carriers.

Stable cell transfectants of all these protein constructs in PC12, N2A, and NIH3T3 cells were obtained with Lipofectamine (Invitrogen). Transfection with G418 at 800 μg/ml. Either a pool of transfected cells or individual cloned cell lines (obtained by serial cloning and colony picking) were used in the present studies with no significant differences in the results. Transfections were performed with Lipofectamine (Invitrogen) and/or cotransfection.

Stable cell transfectants of all these protein constructs in various cell lines, such as IκBα, were obtained from BD Biosciences (clone JL-8) and used at various concentrations: 10–500 ng/ml, 10–100 μg/ml, respectively.

Flow Cytometry and Fluorescence Microscopy—Cells were detached from the plates, washed once in cold phosphate-buffered saline by centrifugation at 1000 × g for 5 min, and resuspended in phosphate-buffered saline. Cell viability was routinely assayed by staining with propidium iodide (1 μg/ml) and was always below 10% in all experimental conditions tested in this study. Fluorescence was determined in a FACSort flow cytometer (BD Biosciences) and analyzed with CellQuest software. Gating was initially performed in forward scatter versus side scatter dot-plots. For fluorescence microscopy cells were plated over glass coverslips, subjected to different treatments, fixed with paraformaldehyde, mounted, and examined with a Leica confocal microscope.

Protein Analysis—Expression of fluorescent proteins was analyzed by Western immunoblotting of total cell extracts prepared by direct lysis of cells in SDS loading buffer and separation by 12% SDS-PAGE. Anti-EGFP monoclonal antibodies were obtained from BD Biosciences (clone JL-8) and used at...
1:5000 dilution. Anti-c-Jun (H-79) polyclonal antibody and anti-phospho-c-Jun (KM-1) monoclonal antibody were obtained from Santa Cruz Biotechnology and used at 1:1000 dilution. The rabbit antiserum used against Fos family (RR26/8) was kindly provided by Dr. Juan Miguel Redondo (17) and used at 1:1000 dilution. Blots were developed with peroxidase-labeled goat anti-mouse (or anti-rabbit) antibody from Bio-Rad at 1:4000 dilution by chemiluminescence method. Controls for the amounts of loaded proteins were performed with anti-tubulin antibodies (Sigma). Blots were analyzed by quantitative densitometry using Quantity One software (Bio-Rad). Luciferase activity was assayed with the Dual-Luciferase reporter assay system from Promega according to the manufacturer's protocol. Results are expressed as the means +/− S.E. for the quotient of firefly luciferase and Renilla luciferase activity.

RNA Analysis—Total RNA was extracted by the method of Chomczynski and Sacchi (18) using TRIzol (Invitrogen). Isolated RNA was treated for 45 min at room temperature with DNase I (amplification grade) from Invitrogen to eliminate traces of plasmidic and genomic DNA. The RNA integrity was assessed by RNA chips using the RNA 6000 Nano kit. 1–2 μg of total RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Semiquantitative estimation of GFP RNA levels was performed by RT-PCR using the following oligonucleotides: forward, 5’-GAACGGCATCAAGGT-GAAC-T-3’; reverse, 5’-GAACCTC-CAGCAGGACCATTG-3’. Control RT-PCR was performed with the inclusion in the same RT-PCR of actin oligonucleotides: forward, 5’-AGCCATGTACGTAGCATTCC-3’; reverse, 5’-CTCTCAGCT-GTGTTGGTGA-A-3’. After cDNA synthesis, PCR was performed with Taq polymerase (Bioline) with an initial incubation at 95 °C for 7 min to activate the polymerase. This initial cycle was followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. This extension consisted of 72 °C for 10 min. The products were analyzed by agarose gels and stained with ethidium bromide.

For direct visualization and quantification of mRNAs, Northern blot analysis was performed. 5–10 μg of total RNA was separated on a 1% agarose denaturing gel and transferred onto charged nylon membranes (Zeta-Probe GT, Bio-Rad), and the membranes were UV light cross-linked and stained with methylene blue for ribosomal 28 and 18 S visualization. DNA probes were generated from the purified inserts of the following plasmid constructs after restriction endonuclease digestion (enzymes indicated in parentheses): pEYFP C-1 (NheI/HindIII) from Clontech, pCMV Sport6 mouse glyceraldehyde-3-phosphate dehydrogenase (I.M.A.G.E. Clone ID 6494145) (SalI/NotI), and pGEM rat protein-disulfide isomerase (PstI) as described previously (19). The purified inserts (50 ng) were labeled with 50 μCi of [α-32P]dCTP using the Rediprime™ II DNA Labeling System (GE Healthcare) and purified using Illustra NICK columns (GE Healthcare) according to the manufacturer’s instructions. The same filter was probed, washed, and hybridized successively with the above indicated cDNA probes. For developing, the blots were exposed to a storage phosphor screen (2–4 h) and scanned in a Typhoon Trio Imager (GE Healthcare); quantification was performed with ImageQuant TL or Quantity One software from Bio-Rad.

Quantitative real time PCR (qPCR) was performed using a 7900 HT Fast Real-Time PCR System with Fast SYBR Green Master Mix (Applied Biosystems) using the standard protocol for PCR Fast. For the target gene (GFP) the following oligonucleotides were used: forward, 5’-GGGCACAAGCTGGAGTA-
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FIGURE 2. Fluorescence microscopy analysis of EYFP-C-Cot transiently transfected PC12 and NIH3T3 cells treated with different doses of proteasome inhibitors. Upper panels show EYFP fluorescence, and lower panels show EYFP-C-Cot fluorescence. Cells were treated with different doses of proteasome inhibitors. Similar results were obtained in PC12 and NIH3T3 cells (Fig. 2). The results presented are similar to those reported by other authors with different unstable fluorescent proteins (8, 5, 9, 10). Other protease inhibitors, Pefabloc (100 μM), had no effect (data not shown). These results indicate the specificity of the effect of proteasome inhibitors. In general, three main cellular processes, transcription, translation, and degradation, are responsible for the steady-state levels of proteins in the cell. Total protein translation was not significantly affected by treatment of the cells with proteasome inhibitors as measured by incorporation of [35S]Met/Cys into total proteins; identical results were obtained by other authors under experimental conditions similar to the ones used in this study (21). When we checked transcription, to our surprise the addition of actinomycin D (ActD) blocked the increase in the amount of the EYFP-C-Cot reporter protein produced by proteasome inhibitors (data not shown). Similar results were obtained with stable transfected cell lines, either transfected pools or clonal cell lines of PC12 and NIH3T3 cells.

Transcriptional Regulation of CMV-driven Fluorescence Reporter Constructs—The above results could be a particular property of our reporter EYFP-C-Cot construct, so we decided to analyze other GFP reporters, EGFPd2 (EGFP fused with the C-terminal amino acids 422–461 from ornithine decarboxylase), a direct substrate of the 26 S proteasome (not requiring ubiquitylation for its degradation), and GFPu (GFP-C1 construct, ACKNWFSSL8HVF1HL). To that end, PC12, NIH3T3, and N2A stable transfectants of this reporter construct were obtained in PC12 and NIH3T3 cells, and our aim was to study the possible differential regulation of proteasome activity in cells of neural and non-neural origin. Untreated cells showed little fluorescence, and after addition of proteasome inhibitors a dose-dependent increase in the amount of the reporter fusion protein was invariably obtained in parallel with increase levels of the reported protein as demonstrated by Western blot analysis (Fig. 1). These results were also confirmed by direct fluorescence microscopy observation of 3–4 pools of selected cells and several individual clones were analyzed, and the results obtained were similar to those presented above for EYFP-C-Cot and in agreement with previous reports using these constructs (8–10). As an example, the results for the EGFPd2 in PC12 cells are presented. Fluorescence intensity per cell increase in a dose-dependent manner. Low magnification confocal images (×200) are shown. All pictures were taken with the same settings of the confocal microscope.

CAACT-3′; reverse, 5′-TCCTTGACGCCTCTGCA-3′. The oligonucleotides of the genes for normalization were: TATA-binding protein: forward, 5′-TGCCAGCGCTCGTCTCATAGA-3′; reverse, 5′-GTCCGATACGGCCAAATCC-3′; glyceraldehyde-3-phosphate dehydrogenase: forward, 5′-TGCCAGCCTCGTCTCATAGA-3′; reverse, 5′-GTCCGATACGGCCAAATCC-3′; and TATA-binding protein: forward, 5′-TCCTTGACGCCTCTGCA-3′; reverse, 5′-CCCTTGACCGCCAGCATTT-3′. After validation of similar amplification efficiencies for target and reference genes (100 ± 5%), the relative expression levels were analyzed by the ΔΔC_T comparative method (20) from three different experiments. The C_T ranges for the analyzed genes were between 15–23 cycles. Results are expressed as -fold changes, taking the control (no treatment) as a reference value of 1 plus or minus the corresponding range.

RESULTS

EYFP C-terminal Cot/Tpl2 UPS Reporter—We have reported that the C terminus of Cot/Tpl2 kinase, when transfected to EYFP renders the fusion protein unstable, and it is degraded by the proteasome (12). We used this construct (EYFP-C-Cot) as a possible new fluorescent reporter to monitor proteasome activity in living cells. Transient cell transfectants of this reporter construct were obtained in PC12 and NIH3T3 cells, and our aim was to study the possible differential regulation of proteasome activity in cells of neural and non-neural origin. Untreated cells showed little fluorescence, and after addition of proteasome inhibitors a dose-dependent increase in the amount of the reporter fusion protein was invariably obtained in parallel with increase levels of the reported protein as demonstrated by Western blot analysis (Fig. 1). These results were also confirmed by direct fluorescence microscopy observation of 3–4 pools of selected cells and several individual clones were analyzed, and the results obtained were similar to those presented above for EYFP-C-Cot and in agreement with previous reports using these constructs (8–10). As an example, the results for the EGFPd2 in PC12 cells are presented. Fluorescence intensity per cell increase in a dose-dependent manner. Low magnification confocal images (×200) are shown. All pictures were taken with the same settings of the confocal microscope.
cence-activated cell sorter scan profiles are presented in Fig. 3a, and the quantification of EGFD2 is presented in Fig. 3b, upper panel. The corresponding amounts of EGFD2 mRNA and protein (Fig. 3b) were in parallel with the cell fluorescence results. The addition of ActD to control cells (Fig. 3b, lane 3) produced a small reduction in the basal expression of the protein, whereas cycloheximide (CHX; Fig. 3b, lane 4) as expected substantially reduced the steady-state levels of the fluorescent reporter protein. These results demonstrate that inhibition of protein synthesis produces the rapid degradation of the EGFPd2 protein.

Treatment with proteasome inhibitors (MG132, lactacystin, or epoxomicin) produced a strong increase (Fig. 3b) in cell mean fluorescence intensity in parallel with the increase in the amount of mRNA and protein levels (Fig. 3b, lanes 5, 8, and 11) as determined by semiquantitative RT-PCR and immunoblotting, respectively. The parallel increase of fluorescence, mRNA, and protein levels produced by treatment with proteasome inhibitors was abolished (or strongly reduced) when either ActD or CHX was added together with proteasome inhibitors (Fig. 3b, lanes 6, 7, 9, 10, 12, and 13). Time course experiments demonstrated that the effects are clearly observed after 4 h of treatment and plateau in a dose-dependent manner after 12 h of treatment. Those results were also evident by direct confocal cell fluorescence analysis as illustrated in Fig. 4 for the experiments with MG132.

The interpretation of the results presented in Fig. 3 can be misleading because of the instability of the EGFPd2 protein, but they predict that similar effects should be observed with the stable EGFP (or EYFP). The results for EYFP are presented in Figs. 5 and 6, clearly demonstrating results similar to those obtained with the unstable EGFPd2.

To get a more direct demonstration of the effect of proteasome inhibitors on mRNA, mRNA levels of
EYFP were analyzed by Northern blot, and the results are presented in Fig. 7. Glyceraldehyde-3-phosphate dehydrogenase (a cytoplasmic and nuclear localized glycolytic enzyme) and protein-disulfide isomerase (a lumenal endoplasmic reticulum enzyme) mRNA levels were used as internal controls. Proteasome inhibitors produced (Fig. 7) a clear and significant increase in the mRNA expression levels of EYFP suppressed by co-treatment with transcriptional (ActD) and translational (CHX) inhibitors. As expected, similar results were also obtained with EGFPd2 construct (as shown for MG132 in supplemental Fig. 3). Those changes in the mRNA levels for EGFPd2 and EYFP were confirmed by qPCR analysis (supplemental Fig. 3). The conclusion from these experiments is that proteasome inhibitors are able to activate the transcription of the reporter gene constructs and therefore produce an increase in the amount of mRNA and protein that is the main cause of the increase of the cell fluorescence and protein amounts of unstable (short half-life) as well as stable (long half-life) fluorescent proteins. Similar results were obtained with the EGFPu (as shown for MG132 in supplemental Figs. 4 and 5). Alternative transcriptional (α-amanitin) and translational (emetine) inhibitors also produced similar results (supplemental Fig. 6). Although we analyzed several different clones (and pools) of stable cell transfectants and the response was similar to that shown in Figs. 3 and 5, it could be that proteasome inhibitors are able to release the transcriptional silencing of the cell-integrated DNA constructs in the stable cell lines because silencing has been reported to occur for the CMV promoter in stable transfectants (22, 23). Therefore, the same types of experiments were performed in transiently transfected cells (PC12, N2A, and HeLa cells) that are unlikely to show these possible epigenetic modifications because it has been demonstrated that the CMV-GFP plasmid
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remains unmethylated for a period up to 96 h in transiently transfected cells (22, 23), and it is transcribed and observed by Northern blot analysis of the effect of proteasome, transcription factors that are cell-specific and responsive to different stimuli and stress: NFκB/Rel (25–27), AP1 (28), ATF/CREB (26, 29, 30), YY1, and NF-1 (31–33). To explore the possible transcription factors responsible for CMV up-regulation by proteasome inhibitors, we used competition experiments in transiently transfected HeLa cells. First we performed experiments similar to those described above for murine cells with transiently transfected HeLa cells (supplemental Fig. 7) for both stable EYFP and unstable GFPu. Treatment of transfected HeLa cells with proteasome inhibitor (MG132) increased the amount of mRNAs (as measured by qPCR) and protein levels, and again, as shown above with murine cells, co-treatment with ActD and CHX suppressed the increases observed (supplemental Fig. 7). Once established that human cells behave similarly to murine cells, the DNA dose dependence of both unstable (GFPu) and stable (EYFP) proteins and its response to proteasome inhibition with MG132 were evaluated in transiently transfected HeLa cells. Results are presented in supplemental Fig. 8. MG132 treatment increased GFPu cell contents, measured both by cell fluorescence and immunoblotting, in an almost linear manner. In contrast, the MG132 effect on EYFP cell contents leveled off (both by cell fluorescence and immunoblotting), indicating saturation of the transfected cells for EYFP expression; accordingly the degree of MG132 activation decreased as expression saturation was reached. Once we had defined the experimental setting in HeLa cells, three different luciferase reporters driven by AP1, CREB, and NFκB binding sites were selected to perform competition experiments. In the absence of proteasome inhibitors, the expression levels of the GFPu and EYFP proteins from their respective plasmids was not affected by co-infection with those luciferase reporter plasmids; similarly to pcDNA3.1 or pEF-BOS (supplemental Fig. 8). Therefore, the study of competition for the expression of luciferase reporter mRNA by proteasome inhibitors was done by co-transfecting human CMV promoter packaged DNA of GFPu and EYFP with a 10-fold excess of either empty vector as control or the AP1 binding sites as a 10-fold excess of empty pcDNA3.1 (one AP1 binding site) vector is inefficient as a competitor (see Fig. 8 and supplemental Fig. 8) to prevent the up-regulation of GFP reporter by proteasome inhibitors or basal expression (supplemental Fig. 9). In contrast, a 30-fold excess of AP1 binding sites, as provided by 10-fold higher amounts of the AP1 luciferase reporter (see Fig. 8d) showed that MG132 treatment only increased firefly luciferase expression in the case of the AP1 luciferase reporter. These results also suggest that effective transcriptional competition under these experimental conditions requires a high amount of AP1 binding sites as a 10-fold excess of empty pcDNA3.1 (one AP1 binding site) vector is inefficient as a competitor (see Fig. 8 and supplemental Fig. 8) to prevent the up-regulation of GFP reporter by proteasome inhibitors or basal expression (supplemental Fig. 9). In contrast, a 30-fold excess of AP1 binding sites, as provided by 10-fold higher amounts of the AP1 luciferase reporter with three AP1 binding sites in tandem, can efficiently prevent the transcriptional activation of the GFP reporter by proteasome inhibitors and are in perfect agreement with the results presented in Fig. 8d showing that MG132 treatment only increased firefly luciferase expression in the case of the AP1 luciferase reporter. These results also suggest that effective transcriptional competition under these experimental conditions requires a high amount of AP1 binding sites as a 10-fold excess of empty pcDNA3.1 (one AP1 binding site) vector is inefficient as a competitor (see Fig. 8 and supplemental Fig. 8) to prevent the up-regulation of GFP reporter by proteasome inhibitors or basal expression (supplemental Fig. 9). In contrast, a 30-fold excess of AP1 binding sites, as provided by 10-fold higher amounts of the AP1 luciferase reporter with three AP1 binding sites in tandem, can efficiently prevent the transcriptional activation of the GFP reporter by proteasome inhibitors. Avoiding Transcriptional Regulation in the Study of Protein Degradation by UPS—The above results point clearly to the need for experimental conditions where the RNA of the reporter protein is not transcriptionally up-regulated by the use of proteasome inhibitors to measure protein degradation. To
that end and based on the results shown above, it is clear that an increase in the amounts of unstable GFP proteins is needed to accurately measure its degradation because of the low steady-state levels of the unstable proteins. The simplest way is to add proteasome inhibitors for a short period of time and get a sufficient amount of the reporter protein to easily follow its degradation. For this type of experiment a reversible proteasome inhibitor, like MG132, that can be washed out from the cell has to be used. Then the addition of CHX will produce protein synthesis inhibition, allowing the degradation of the unstable reporter protein to be observed. Because CHX is present, the addition of new proteasome inhibitor in the presence of CHX will not produce an up-regulation of the CMV promoter (as it has been shown above). As a consequence we can safely determine the half-life of the unstable protein without interference of transcriptional and translational input to the unstable protein pool in the cell. One such experiment is presented in Fig. 10 for the EGFPd2 unstable protein. The EGFPd2 protein was degraded with an estimated half-life of 4–5 h in the presence of CHX, and proteasome inhibitors prevented its degradation without affecting the expression of the corresponding mRNA as judged by qPCR (Fig. 10). Under these experimental conditions any compound or gene product can be tested for its effect on proteasome activity in the cell without the inconvenience of transcriptional regulation of the CMV promoter.

**FIGURE 8.** Transcription factors implicated in the activation of CMV fluorescent reporters by proteasome inhibitors. HeLa cells were transiently transfected with 50 ng of the corresponding pCMV GFPu (a) and pCMV EYFP (b) together with 950 ng of the indicated plasmids: empty pCDNA3.1, empty pEF-BOS, and AP1, CRE, NFκB luciferase reporters. Transfected cells were untreated (control) or treated with MG132 (10 μM) for 12 h. Total cells extracts were analyzed by immunoblots and autoradiograms to show -fold changes in total cell fluorescence intensity analyzed by flow cytometry. A shows representative Western immunoblot of the expression of the fluorescent proteins. d, HeLa cells were transfected with a mixture of DNAs containing 100 ng of the indicated firefly luciferase reporters, 50 ng of basal NFκB, and αB luciferase plasmid, and 850 ng of empty pcDNA3.1 vector. Transfected cells were untreated (control) or treated with MG132 (10 μM) for 12 h, and luciferase was assayed as described under “Experimental Procedures.” The graph shows -fold changes in the quotient of firefly/RENilla luciferase result in three different experiments. Tub, tubulin; CRE, cAMP-responsive element binding protein; c-Fos, c-Jun, and p-c-Jun.

**FIGURE 9.** Effect of MG132 treatment of HeLa and PC12 cells on c-Jun and c-Fos expression. HeLa or PC12 cells were untreated (−) or treated (+) with 10 μM MG132 for 12 h. Total cells extracts were analyzed by immunoblots with the indicated antibodies to detect total c-Fos and c-Jun and the phosphorylation of c-Jun (P-c-Jun) as described under “Experimental Procedures.”

**Discussion**

We have presented experimental evidence showing that transcriptional regulation of the CMV promoter by proteasome inhibitors plays a substantial role in the activation of AP1 transcription factor by stabilization of both Jun and Fos transcription factors limiting the transcriptional output but also in recycling of transcriptional complexes on chromatin to facilitate multiple rounds of transcription (37–39). Proteasome inhibitors, as a consequence, have been reported to have multiple effects depending on the context of the specific promoter under study. Inhibition of proteasomal degradation increases transcriptional activity of some, but not all, steroid hormone receptors (40), produces a heat-shock response with an increase of cytoplasmic and endoplasmic reticulum chaperones (41) mediated by HSF1–3 (42) and favoring proteostasis (43), and promotes an increase in the global levels of trimethyl histone H3K4 and phosphorylated RNA polymerase II forms that affect global gene transcription (44). In the context of the CMV promoter it would be expected that proteasome inhibitors could prevent NFκB/Rel activation by inhibition of IκB degradation (45), activate AP1 by stabilization of both Jun and Fos transcription factors and direct activation of stress kinases (34–36), promote the activation of CREB by inhibition of its proteasomal degradation (47), and stabilize YY1 that may behave as an activator or as a repressor depending on the cell context (48). Our experimental strategy based on transcriptional competition experiments with specific transcriptional reporter constructs instead of using stress kinase inhibitors or other interference treatments that are always subject to nonspecific effects gave unequivocal experimental evidence that the AP1 transcription factor is the main transcription factor responsible for the observed increase in protein levels of both unstable and stable GFPs after treatment with proteasome inhibitors due to the stabilization of
c-Jun and c-Fos transcription factors and the phosphorylation of c-Jun, although still other transcription factors that bind to the CMV promoter can also participate. Actually the transcriptional activation of CMV promoter by proteasome inhibitors has already been described by other groups (49, 50). The fact that the increase of mRNA expression of CMV-driven constructs by proteasome inhibitors was blocked by ActD (and α-amanitin) is not surprising; more striking could be that with CHX, a protein synthesis inhibitor, that also induces transcriptional up-regulation of the CMV-driven constructs. Although CHX is an inhibitor, one should remember that it can affect gene transcription by facilitating the degradation of labile transcription factors, either by blocking the synthesis of activators or repressors, or by preventing its synthesis. In fact, Li et al. (50) also reported that CHX blocks the transcriptional activation of CMV promoter constructs to a degree proportional to what is reported here. As with the over-expression of CMV promoter by proteasome inhibitors, the level of protein synthesis directly or indirectly affects post-translational modification of the transcriptionally active machinery involved in CMV transcription.

The main message of the present work is that the measurement of UPS activity in cells or in vivo in transgenic animals with unstable proteins requires the compulsory demonstration that the level of the mRNAs for those protein constructs is not affected by the experimental treatments applied to the cells or animals. Simple conclusions based on fluorescence and/or immunoblots of the reporter protein can give rise to unjustified and misleading conclusions about proteasome activity. The misleading conclusion may even look substantiated by the experiments. Under saturating amounts of transfected DNA (see supplemental Fig. 8) proteasome inhibitor treatment will not affect the expression levels of the stable GFP constructs measured by protein amounts or fluorescence (as expected for a stable protein) because the cell expression system is saturated, whereas at the same DNA doses of the unstable GFP construct, the levels of the unstable GFP will be nicely increased by proteasome inhibitors (as expected for an unstable protein). This general statement is perfectly exemplified in the present case for the CMV promoter where the results of proteasome inhibi-
Legends to supplementary Figures

Legend to Supplementary Fig. 1. Effect of different protease inhibitors on the expression of EYFP-Ct-Cot in PC12 cells. PC12 cells were untransfected or transiently transfected with EYFP-C-cot and untreated (control) or treated with different protease inhibitors E64-d (50 µM), Pefablock (100 µM), leupeptin (200 µM), MG132 (10 µM) or lactacystin (10 µM) for 12h, as indicated. Graph show the relative fold change on cell fluorescence mean intensity ± S.E.M. from three different experiments. Immunoblots show the expression of EYFP-C-cot in untransfected, control cells (transfected cells, no treatment) and cells transfected and treated with the different protease inhibitors, as indicated. Note that only treatment with proteasome inhibitors (MG132 and lactacystin) produce an increase in fluorescence and protein expression levels.

Legend to supplementary Fig. 2 Northern blot analysis of the effect of proteasome, transcription and translation inhibitors on the mRNA expression of unstable EGFPd2. PC12 cells untransfected or stably transfected with unstable EGFPd2 were treated for 12h with proteasome inhibitors (10µM MG132) in the absence or in the presence of 500ng/ml actinomycin D (ActD), or 20 µg/ml cycloheximide (CHX), as indicated. Total RNA was isolated from cells and analyzed by Northern blot. Membranes of transferred RNAs were stained with methylene blue (28S and 18S RNAs) and successively hybridized with 32P-labeled probes for EYFP, GAPDH and PDI. A representative experiment is presented. Graph shows the relative changes in the amount of mRNA for EYFP quantitated using either GAPDH (white bars) or PDI (grey bars) mRNAs as controls.

Legend to supplementary Fig. 3. qPCR analysis of the effect of proteasome, transcription and translation inhibitors on the mRNA expression of unstable EGFPd2 and stable EYFP in PC12 cells. PC12 cells stably transfected with unstable EGFPd2 or stable EYFP constructs where untreated (control) or treated for 12h with proteasome inhibitors (10µM MG132) in the absence or in the presence of 500ng/ml actinomycin D (ActD), or 20 µg/ml cycloheximide (CHX), as indicated. Total RNA was isolated and analyzed by qPCR, as described under “Experimental Procedures”. Graphs show the relative fold change and the corresponding ranges (upper and lower bars) as calculated from three different experiments from –ΔΔCt values.

Legend to supplementary Fig. 4. Effect of proteasome, transcription and translation inhibitors on the expression of unstable GFPu. PC12 cells untransfected or stably transfected with either unstable EGFPd2 (a) or stable EGFP (b), were treated for 12h with proteasome inhibitors (10µM MG132) in the absence or in the presence of 500ng/ml actinomycin D (ActD), or 20 µg/ml cycloheximide (CHX), as indicated. Panel a), graph of cell fluorescence mean intensity analyzed by flow cytometry and expressed as mean ± S.E.M from three different experiments each done by triplicate. Lower pictures show representative experiments of mRNA (EGFP and actin, used as control) analyzed by 1.5% agarose gel and ethidium bromide staining of RT-PCRs products obtained from the respective total cell mRNAs samples; and protein levels (EGFP and tubulin, used as control) by Western and immunoblot with anti-EGFP and anti-tubulin antibodies. Panel b), Total RNA was isolated from cells after the corresponding treatments and analyzed by Northern blot. Membranes of transferred RNAs were stained with methylene blue (28S and 18S RNAs) and successively hybridized with 32P-labeled probes for EYFP, GAPDH and PDI. Graph shows the relative changes in the amount of mRNA for EGFP quantitated using either GAPDH (white bars) or PDI (grey bars) mRNAs as controls.

Legend to supplementary Fig. 5. Fluorescence microscopy analysis of unstable GFPu transfected PC12 cells in response to proteasome, transcriptional and translational inhibitors. Cells stably expressing GFPu were untreated (control) or treated as indicated for 12h with
MG132, ActD and CHX or the combinations indicated. Living cells were examined under confocal microscopy with medium magnification (x 400), fluorescence images are superimposed over phase-contrast images of the cells. All fluorescent images were captured with the same settings of the confocal microscope.

Legend to Supplementary Fig. 6. Effect of proteasome, transcription and translation inhibitors on cell fluorescence mRNA and protein levels of PC12 cells stably transfected with EYFP and GFPu. Left panels, EYFP; right panels, GFP. Upper panels (both sides), graphs of cell fluorescence mean intensity analyzed by flow cytometry and expressed as mean ± S.E.M from three different experiments, each run in triplicate. Lower panels (both sides) show representative experiments of the levels of mRNA analyzed by 1.5% agarose gel and ethidium bromide staining of RT-PCRs products obtained from the respective total cell mRNAs samples; and protein (GFP and tubulin, used as control) by immunoblotting with anti-EGFP and anti-tubulin antibodies. PC12 cells stably transfected cell lines (2-7) where untreated (control, 2) or treated for 12h with proteasome inhibitors (10µM MG132, in the absence or in the presence of 5 µg/ml of α-amanitine or 7.5 µg/ml emetine, as indicated (3-7).

Legend to supplementary Fig. 7. Effect of proteasome, transcription and translation inhibitors on mRNA and protein abundance of Hela cells transiently transfected with EYFP and EGFPd2. Hela cells transiently transfected with EYFP or EGFPd2 constructs were untreated (control) or treated for 14h with proteasome inhibitors (10µM MG132) in the absence, or in the presence of 500 ng/ml actinomycin D (ActD), or 20 µg/ml cycloheximide (CHX), as indicated. Total RNA was isolated and analyzed by qPCR, as described under “Experimental Procedures”. Graphs show the relative fold change and the corresponding ranges (upper and lower bars) as calculated from three different experiments from –ΔΔCt values. Lower panels show protein expression levels for EYFP and EGFPd2, and tubulin, used as control, by Western and immunoblot with anti-EGFP and anti-tubulin antibodies.

Legend to supplementary Fig. 8. DNA dose-dependent expression of fluorescent proteins in Hela cells. Hela cells were transiently transfected with increasing amounts of the corresponding pCMV GFPu (a) and pCMV EYFP (b) together with the indicated amounts of the following plasmids: empty pcDNA3.1, and AP-1, CREB, or NFκB luciferase reporters. Cell fluorescence was analyzed by flow cytometry. Results presented are average from two different experiments.

Legend to supplementary Fig. 9. DNA dose-dependent expression of fluorescent proteins in Hela cells. Hela cells were transiently transfected with increasing amounts of the corresponding pCMV GFPu (a) and pCMV EYFP (b) together with the indicated amounts of the following plasmids: empty pcDNA3.1, and AP-1, CREB, or NFκB luciferase reporters. Cell fluorescence was analyzed by flow cytometry. Results presented are average from two different experiments.
Supplementary Figure 1
Supplementary Fig. 2
Supplementary Fig. 3
Supplementary Fig. 4
Supplementary Fig. 5
### Supplementary Figure 6

|          | EYFP | mRNA | Protein | β-actin | mRNA | Protein | GFPu | mRNA | Protein |
|----------|------|------|---------|--------|------|---------|------|------|---------|
| Control  |      |      |         |        |      |         |      |      |         |
| 2        |      |      |         |        |      |         |      |      |         |
| 3 α-Amanitine |      |      |         |        |      |         |      |      |         |
| 4 Emetine |      |      |         |        |      |         |      |      |         |
| Untransfected |      |      |         |        |      |         |      |      |         |
| 1        |      |      |         |        |      |         |      |      |         |
| 2        |      |      |         |        |      |         |      |      |         |
| 5 α-Amanitine |      |      |         |        |      |         |      |      |         |
| 6 Emetine |      |      |         |        |      |         |      |      |         |
| 7        |      |      |         |        |      |         |      |      |         |

### Mean Fluorescence

|          |          |          |
|----------|----------|----------|
|          |          |          |
|          |          |          |

**EYFP**

**GFPu**

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Supplementary Fig. 7

**EYFP mRNA qPCR**

- Control
- ActD
- Chx
- MG132
- MG132 + ActD
- MG132 + Chx

**GFPu mRNA qPCR**

- Chx
- MG132
- MG132 + ActD
- MG132 + Chx

**Fold Change**

- EYFP mRNA qPCR
- GFPu mRNA qPCR

**Western Blot**

- EYFP
- GFPu
- Tubulin

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Supplementary Fig. 8
Supplementary Fig. 9
Cytomegalovirus Promoter Up-regulation Is the Major Cause of Increased Protein Levels of Unstable Reporter Proteins after Treatment of Living Cells with Proteasome Inhibitors

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