Proteasome Inhibitors Reduce Luciferase and β-Galactosidase Activity in Tissue Culture Cells*

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Reporters are commonly used in cell biology to study transcriptional activity of genes. Recently, reporter enzymes in combination with compounds that inhibit proteasome function have been used to study the effect of blocking transcription factor degradation on gene activation. While investigating the effect of proteasome inhibition on steroid receptor activation of the mouse mammary tumor virus (MMTV) promoter, we found that treatment with proteasome inhibitors enhanced glucocorticoid activation of the promoter attached to a chloramphenical acetyltransferase (CAT) reporter, but inhibited activation of MMTV attached to a firefly luciferase or β-galactosidase reporter. MMTV RNA levels under these conditions correlated with the promoter activity observed using the CAT reporter, suggesting that proteasome inhibitor treatment interfered with luciferase or β-galactosidase reporter assays. Washout experiments demonstrated that the majority of luciferase activity was lost if the proteasome inhibitor was added at the same time luciferase was produced, not once the functional protein was made, suggesting that the proteasome inhibition interferes with production of luciferase protein. Indeed, we found that proteasome inhibitor treatment dramatically reduced the levels of luciferase and β-galactosidase protein produced, as determined by Western blot. Thus, treatment with proteasome inhibitors interferes with luciferase and β-galactosidase reporter assays, possibly by inhibiting production of a functional reporter protein.

The degradation of most proteins in mammalian cells occurs via the ubiquitin-proteasome pathway (1). In this process, substrate proteins are covalently linked to multiple ubiquitin molecules, which target the protein to the 26 S proteasome for degradation (2). The 26 S proteasome is a 2-MDa multisubunit complex that degrades proteins in an ATP-dependent manner into peptides 3–20 amino acid residues in length (1). The proteasome degrades both short-lived proteins (t1/2 < 3 h) and more stable proteins (t1/2 of hours or days). Since 1994, reagents that inhibit proteasome function have facilitated the investigation of protein turnover by this pathway (1). These inhibitors are now commonly used to study the degradation of proteins from many cellular pathways.

Although one of the proteasome’s major roles is the breakdown of misfolded proteins, it also regulates the levels of potent regulatory molecules such as transcription factors, cell cycle proteins, and tumor suppressor proteins (3). Recently, the relationship between steroid receptor-mediated transcription and receptor degradation has been investigated using proteasome inhibitors in combination with widely available reporter enzymes such as chloramphenical acetyltransferase (CAT) and firefly luciferase (4–6). Reporter enzymes such as firefly luciferase, and β-galactosidase and CAT from Escherichia coli, are commonly used in cellular and molecular biology to study promoter activation of genes in mammalian cells. In general, the activity of these reporters is unaffected by the specific treatment regimen, and interference of the treatment with the reporter itself is generally not considered. However, both firefly luciferase and β-galactosidase can be inactivated under conditions of stress to the cell, such as thermal stress, chemical stress, or oxidative stress (7–9). Thus, the interpretation of these assays can be compromised under certain treatment conditions, such as those inducing stress.

While investigating the effect of proteasome inhibitors on glucocorticoid receptor (GR)-mediated transcription of the mouse mammary tumor virus (MMTV) promoter, we noticed that several of these compounds interfere with both firefly luciferase and β-galactosidase enzymatic activity in tissue culture cells, while CAT activity is unaffected. MMTV RNA levels induced by glucocorticoid and proteasome inhibitor treatment correlated with CAT activity from the MMTV promoter, but not luciferase or β-galactosidase activity. The inhibition of activity occurred primarily if the proteasome inhibitor was added before production of the protein, rather than after formation of the fully functional protein. Further investigation indicated that proteasome inhibitor treatment reduced the levels of both luciferase and β-galactosidase protein, suggesting that these compounds interfere with production of a functional protein. Thus, use of proteasome inhibitors in combination with the luciferase and β-galactosidase reporters may lead to an unexpected reduction of enzymatic activity and interference with interpretation of these assays.

EXPERIMENTAL PROCEDURES

Cell Culture—A1-2 cells were derived from T47D breast cancer cells by stable transfection with pGRneo and MMTV-LTR-luc plasmids as described previously (10). GR2 cells were derived by stably transfecting a GR-neo expression vector into a T47D-based cell line lacking the GR, but containing a stably integrated MMTV-CAT reporter (11). Both A1-2 and GR2 cells were grown at 37 °C with 5% CO2 in modified Eagle’s medium supplemented with 2 mM glutamine, 100 μg/ml penicillin/streptomycin, 10 mM HEPES, and 10% fetal bovine serum and maintained with 1 μg/ml puromycin (GR2) or 160 μg/ml G418 (A1-2). HeLa cells were grown at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 100 μg/ml penicillin/streptomycin, 10 mM HEPES, and 10% fetal bovine serum.

Reporter Assays—For analysis of integrated MMTV-luc and MMTV-
CAT reporters, A1-2 and GR2 cells were plated onto six-well and 100-mm dishes, respectively, and either left untreated or treated with dexamethasone (dex), MG132, or the combination as described in the figure legends to Figs. 1, A, and B, and 2A. CAT activity was determined by a kinetic assay and normalized for total protein (12). Luciferase activity was determined using the Luciferase assay system with Reporter lysis buffer (Promega) according to manufacturer’s instructions and normalized for total protein. For analysis of constitutively expressed luciferase, cells were plated onto six-well dishes and transfected the next day with a pGL3-control plasmid (Promega) using the LipofectAMINE Plus protocol (Invitrogen). The cells were transfected for 3 h, then treated as indicated in the figure legends to Fig. 2, C and D. For β-galactosidase assays, cells were plated and transfected the next day with an MMTV-lacZ construct using the same procedure as for the luciferase assay. β-Galactosidase activity was determined using the β-galactosidase enzyme assay system with reporter lysis Buffer (Promega) according to manufacturer’s instructions. All assays were carried out in either duplicate or triplicate.

Western Blot Analysis—Cells were plated on 100-mm dishes and grown until 80% confluent before preparation of extracts. For β-galactosidase protein determination, cells were transfected with an MMTV-lacZ reporter plasmid the day before treatment. Whole cell lysates were prepared using SDS lysis buffer (7). Cells were briefly resuspended in 1 X Tris-glycine native sample buffer (Invitrogen) containing β-mercaptoethanol, then sonicated to shear DNA. Care was taken to ensure that each lysate was produced from the same number of cells. Samples were boiled for 5 min and separated on 8% NOVEX Tris-glycine gels (Invitrogen), transferred to Hybond-P PVDF membrane (Amersham Biosciences), and immunoblotted with antibodies to luciferase (1:500) (Promega), β-galactosidase (1:500) (Promega), or glyceraldehyde dehydrogenase (GAPDH) (Research Diagnostics Inc.).

RT-PCR—Cells were left untreated or treated as described in the figure legends to Fig. 1C. Total cellular RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized as described previously (13). For PCR of the cDNA, two separate reactions were run for each experimental condition. The cDNA was combined with 5 units of Tth DNA polymerase in a final volume of 50 μl. The PCR mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 100 μM concentration of each dNTP, 10 pmol of MMTV-619, and 0.25 pmol of MMTV-22. MMTV-22 was end-labeled with T4-poly nucleotide kinase to generate a 32P-labeled single-stranded primer. Human β3-microglobulin was similarly amplified using sequences previously described (13). PCR products were analyzed on 8% polyacryl amide gels and exposed to Molecular Dynamics PhosphorImager screens or autoradiography film for analysis.

RESULTS

Proteasome Inhibitors Interfere with Luciferase Activity—To investigate the effect of the proteasome inhibitor, MG132, on glucocorticoid-activated transcription, we used the well characterized steroid-responsive MMTV promoter. Using a similar MG132 dose and time course previously used by others (5, 6), we observed the same amplification of glucocorticoid-induced transcription in the T47D-based human breast cancer cell line, GR2, as has previously been reported in HeLa cells (6). The GR2 cell line contains the GR and a stably integrated MMTV-CAT reporter construct (Fig. 1A). Treatment with the synthetic glucocorticoid, dex, activated the promoter, and MG132 enhanced this activation. However, using a second T47D-based cell line containing the GR and a different reporter-promoter construct, MMTV-CAT, we observed contradictory results. In contrast to the enhanced activation of MMTV-CAT by MG132, treatment with this proteasome inhibitor decreased dex activation of the MMTV-CAT reporter (Fig. 1, A and B, compare lanes 2 and 4). This inactivation was not limited to the compound, MG132, because the proteasome inhibitors lactacystin and proteasome inhibitor I had the same inhibitory effect.2

To determine whether the luciferase or CAT activity reflected the actual RNA being produced in the presence of proteasome inhibitors, we analyzed levels of MMTV-luciferase RNA after dex and MG132 treatment (Fig. 1C). In contrast to the observed luciferase activity, MG132 enhanced the dex-induced increase in MMTV-luciferase RNA (Fig. 1C). We observed an identical RNA profile in GR2 cells containing MMTV-CAT (14). Thus, RNA production from MMTV-luciferase matched the activation seen from the CAT, but not luciferase reporter. It appeared that proteasome inhibitor treatment interfered with luciferase activity under the treatment conditions of our assay, but did not affect production of RNA. We next investigated at what stage of luciferase production the inhibition might occur.

Proteasome Inhibitors Interfere with Production of Luciferase Protein—To determine whether luciferase inactivation by proteasome inhibitors occurred during or after luciferase protein production, we took advantage of the steroid-inducible production of luciferase via the MMTV promoter (Fig. 2A). To study the inactivation of luciferase during protein production, cells were treated for 8 h with dex in the absence (lane 2) or presence (lane 3) of MG132. To study the effect of MG132 on luciferase after the protein was produced, cells were treated for 8 h with dex and then the steroid was removed to block further production of luciferase. The cells were then either left untreated (lane 4) or treated with MG132 (lane 5). Compared with the dex-only controls, co-treatment led to a loss of 70% of luciferase activity (compare lanes 2 and 3), while post-treatment reduced activity by 25%. Note that some loss of luciferase activity is expected after steroid is removed, because the half-life of lucif-
erase is \(-3\) h (compare lanes 2 and 4) (15). Therefore, the majority of luciferase inactivation occurs if the proteasome inhibitor is present when luciferase protein is initially produced.

If proteasome inhibitor treatment interferes with the early stages of luciferase protein formation, then the levels of luciferase protein should be reduced by this treatment in parallel with the loss of activity. We therefore examined luciferase protein levels by Western blot using whole cell SDS lysates with an antibody to luciferase or GAPDH. C, T47D cells were transiently transfected with a pSV-luciferase (pGL3) vector constitutively expressing luciferase and either untreated or treated with MG132 (1 \(\mu\)M) for 24 h. Luciferase activity was determined and normalized against total protein. D, HeLa cells were transiently transfected and analyzed for luciferase activity as described in the legend to C.

Therefore, it appears that the loss of luciferase activity is at least partly due to a reduction in luciferase protein levels.

We then confirmed that inactivation of luciferase was not a promoter or steroid-dependent phenomenon. Luciferase produced from a plasmid constitutively expressing luciferase (pSV-luciferase) was also inactivated by proteasome inhibitor treatment in both T47D and HeLa cells (Fig. 2, C and D). Thus, the reduction in luciferase activity of the MMTV promoter by proteasome inhibitor treatment is not restricted to luciferase produced from a steroid-responsive promoter, but represents a general inhibition of luciferase activity.

**Proteasome Inhibitors Also Inhibit \(\beta\)-Galactosidase Activity**—Both firefly luciferase and \(\beta\)-galactosidase are inactivated by heat shock, ATP depletion, and treatment with ethanol or indomethacin (an anti-inflammatory drug) (7–9). We therefore wanted to determine whether proteasome inhibitors would also interfere with \(\beta\)-galactosidase activity. We observed an identical loss of \(\beta\)-galactosidase activity from the MMTV promoter in the presence of proteasome inhibitors as we did for luciferase activity (Figs. 1B and 3A). Again, this loss of activity correlated with a loss of \(\beta\)-galactosidase protein (Fig. 3B). Levels of a control enzyme, GAPDH, were again unchanged under identical treatment conditions. It therefore appears that production of both luciferase and \(\beta\)-galactosidase protein is inhibited by treatment with proteasome inhibitors, while RNA production is unaffected.

**DISCUSSION**

Reporter enzymes are commonly used to study promoter activity both in vitro and in vivo (16). Enzymes such as luciferase, \(\beta\)-galactosidase, and CAT are not naturally expressed in mammals, making them particularly useful to study gene expression in mammalian cells. These transcriptional reporters are used in many model systems, treatment conditions, and cell types, but rarely is consideration given to the effect of the treatment on the activity of the reporter enzyme itself.

During reporter studies investigating the effect of proteasome inhibition on steroid-mediated transcription of the MMTV promoter, we discovered that proteasome inhibitor
treatment enhanced glucocorticoid-mediated transactivation of an MMTV-CAT reporter (Fig. 1A), but had the opposite effect on the MMTV-luciferase reporter (Fig. 1B). Levels of MMTV RNA induced by steroid and proteasome inhibitor treatment duplicated the CAT activity results, but not luciferase activity (Fig. 1C). Wash-out experiments indicated that maximal inactivation occurred if the proteasome inhibitor was present at the time luciferase was initially produced, rather than after luciferase was made (Fig. 2A). The loss of luciferase activity correlated with a general loss in luciferase protein (Fig. 2B). Importantly, this effect of MG132 on luciferase activity was not limited to the steroid dependent activation of the reporter as it was seen for the constitutively active pSV-luciferase reporter (Fig. 2C). Nor was it specific for T47D cells as a similar effect was observed in HeLa cells (Fig. 2D). The observed inactivation and reduced production of luciferase protein was also observed for the β-galactosidase reporter (Fig. 3). Our results suggest that proteasome inhibitors interfere with luciferase and β-galactosidase production by a post-transcriptional mechanism.

The inactivation of firefly luciferase and β-galactosidase has previously been reported and usually occurs under conditions of cellular stress. Both enzymes are inactivated by heat shock, ATP depletion, and treatment with ethanol or indomethacin (an anti-inflammatory drug) (7–9). In several of these cases, the inactivation occurs because the enzyme is denatured, forming insoluble aggregates that are detected in the insoluble fraction of a cellular lysate (17). The accumulation of abnormal or denatured proteins when proteasome function is blocked is known to activate the heat shock response, indicative of the cell’s response to a stressful condition (18, 19). We initially considered that the cytotoxic nature of proteasome inhibitors might therefore denature luciferase and β-galactosidase, rendering these proteins insoluble, as has been observed under conditions of cellular stress. In these studies, total levels of luciferase and β-galactosidase in SDS lysates are unchanged, although the levels of protein in the insoluble fraction increase when loss of activity is observed (17, 20). In contrast to these studies, we do not observe a loss of solubility, but rather a general reduction in luciferase and β-galactosidase protein levels from SDS lysates, in which both insoluble and soluble luciferase are detectable (Figs. 2B and 3B) (7, 8).

Several mechanisms could explain the loss of luciferase and β-galactosidase protein in response to proteasome inhibitor treatment. One possibility is that the proteasome inhibitor blocks or inhibits translation of these proteins. Second, blocking proteasome function may enhance the proteolysis of newly formed luciferase and β-galactosidase through a proteasome-independent pathway. Third, denaturation of luciferase and β-galactosidase in response to the proteasome inhibitor-mediated cellular stress may render the proteins undetectable by their respective antibodies. However, we consider this third mechanism unlikely, because the same luciferase antibody used in this study readily detects thermally denatured luciferase (21). Thermally denatured β-galactosidase is also readily detectable by its antibody (7).

Regulation of protein production is one mechanism by which cells control intracellular pathways (22). Following stress, protein synthesis is down-regulated in eukaryotic cells, possibly as a protective mechanism that prevents production of toxic proteins (23). It is therefore possible that the cytotoxic effect of proteasome inhibitor treatment results in inhibition of production of luciferase and β-galactosidase proteins. In Jurkat cells, proteasome inhibitors can interfere with translation by promoting degradation of eIF4GI, a eukaryotic translation initiation factor that acts as a molecular bridge between components of the ribosomal initiation complex (24). An interesting question is why the production of luciferase and β-galactosidase may be affected, while production of CAT is not. Translational regulation can occur in an mRNA-specific manner, such that translation of one mRNA may be affected while others are not under identical conditions (25). This may at least partially explain why the production of CAT is unaffected by proteasome inhibitor treatment.

It is also possible that proteasome inhibitor treatment may trigger the proteolysis of newly formed luciferase and β-galactosidase via a ubiquitin-independent degradation pathway. In addition to the ubiquitin-proteasome degradation pathway, the calcium-dependent calpain protease degradation pathway is the second major pathway regulating protein turnover in mammalian cells (25). Perhaps proteasome inhibitor treatment activates calpain-mediated cleavage of luciferase and β-galactosidase. Interestingly, treatment with proteasome inhibitors may trigger apoptosis, and calpain-mediated degradation has been implicated in proteolysis occurring during apoptosis (26, 27).

In summary, we have observed a novel mechanism by which proteasome inhibitor treatment inactivates firefly luciferase and β-galactosidase activity. These results suggest that consideration should be given to the choice of reporter enzyme used when the transcriptional effect of cytotoxic compounds is investigated.

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