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The Protein Degradation Response of *Saccharomyces cerevisiae* to Classical DNA-Damaging Agents

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Genome wide experiments indicate both proteasome- and vacuole-mediated protein degradation modulate sensitivity to classical DNA-damaging agents. Here, we show that global protein degradation is significantly increased upon methyl methanesulfonate (MMS) exposure. In addition, global protein degradation is similarly increased upon exposure to 4-nitroquinoline-*N*-oxide (4NQO) and UV and, to a lesser extent, *tert*-butyl hydroperoxide. The proteasomal inhibitor MG132 decreases both MMS-induced and 4NQO-induced protein degradation, while addition of the vacuolar inhibitor phenylmethanesulfonyl fluoride does not. The addition of both inhibitors grossly inhibits cell growth upon MMS exposure over and above the growth inhibition induced by MMS alone. The MMS-induced protein degradation response remains unchanged in several ubiquitin-proteasome and vacuolar mutants, presumably because these mutants are not totally deficient in either essential pathway. Furthermore, MMS-induced protein degradation is independent of Mec1, Mag1, Rad23, and Rad6, suggesting that the protein degradation response is not transduced through the classical Mec1 DNA damage response pathway or through repair intermediates generated by the base excision, nucleotide excision, or postreplication–DNA repair pathways. These results identify the regulation of protein degradation as an important factor in the recovery of cells from toxicity induced by classical DNA-damaging agents.

Introduction

The proteasome has recently emerged as a target for anticancer chemotherapy. Because cancer cells progress through the cell cycle at a higher rate than most noncancer cells and are often resistant to apoptosis-inducing therapies, one strategy for selectively killing cancer cells is to inhibit proteasomal activity to arrest the cell cycle and increase the susceptibility to apoptosis (*1–3*) (reviewed in ref *4*). Indeed, several preclinical trials have established a selective susceptibility of transformed cells to proteasome inhibitor-mediated apoptosis (*5–8*). Recently, the proteasome inhibitor bortezomib (PS-341 or Velcade) was reported to have positive antitumor effects when administered in combination with several classical chemotherapeutics, including those in the alkylating agent class (*9*). A recent phase II clinical trial demonstrated that the addition of bortezomib to the classical regimen of melphalan plus prednisone results in very high complete recovery rates and increased rates of remission and longevity for elderly multiple myeloma patients (*10*). As cancer therapies that include proteasome inhibitors as part of their regimen emerge, it is important to understand the effects of these new drugs in combination with classical chemotherapeutics such as alkylating agents.

Whole genome-scale studies with *Saccharomyces cerevisiae* reveal that a dynamic alteration of the transcriptome occurs upon exposure to classical DNA-damaging agents (*11–15*). Analysis of such transcriptional changes suggests that the protein degradation machinery may play a role in modulating the toxicity of several agents that are known carcinogens, namely, methyl methanesulfonate (MMS)[1], 4-nitroquinoline-*N*-oxide (4NQO), ultraviolet (UV) irradiation, and *tert*-butyl hydroperoxide (*t*-BuOOH). Numerous genes involved in protein degradation are upregulated upon exposure to MMS, while the protein synthesis machinery is downregulated, suggesting a coordinated turnover of the proteome in response to MMS exposure (*11, 12, 14*). Interestingly, a MMS responsive regulon (the Rpn4 regulon) was identified that includes several protein degradation genes alongside genes in the base excision repair and nucleotide excision repair pathways (*14*). Moreover, genomic phenotyping experiments identified numerous mutants with a compromised ubiquitin−proteasome system or compromised vacuolar function that are sensitive to one or more of these DNA-damaging agents (*15, 16*). The involvement of proteasome and vacuolar function in modulating carcinogen-induced toxicity was further supported by computational mapping of global phenotypic data and transcriptional network data (*13, 15*).

The *S. cerevisiae* 26S proteasome is essential for maintaining normal cell function through regulated protein degradation (*17, 18*). The proteasome constitutes nearly 1% of total cellular protein (*19*), is primarily membrane bound, and is localized mainly in the nuclear envelope−endoplasmic reticulum network (*20, 21*). This large multisubunit complex is composed of a central 20S catalytic core particle that is capped on opposite ends by two 19S regulatory particles, allowing for compart-

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[1] Abbreviations: BER, base excision repair; CSM-Met, complete synthetic media without methionine; DMSO, dimethyl sulfoxide; ERAD, endoplasmic reticulum-associated protein degradation; ESR, environmental stress response; MMS, methyl methanesulfonate; AAG, 3-methyladenine-DNA glycosylase; NER, nucleotide excision repair; 4NQO, 4-nitroquinoline-*N*-oxide; PACE, proteasome-associated control elements; PMSF, phenylmethanesulfonyl fluoride; PRR, postreplication repair; *t*-BuOOH, *tert*-butyl hydroperoxide; TCA, trichloroacetic acid; XPC, xeroderma pigmentosum protein C.

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mentalization and regulation of protease activity. As the terminal macromolecule in the ubiquitin−proteasome pathway, it processively degrades, in an ATP-dependent manner, proteins that have been tagged for degradation by covalent attachment of a polyubiquitin chain (for a review, see refs *17* and *18*). The proteasome primarily degrades specific short-lived proteins in a highly regulated manner as well as general proteins that have been damaged or misfolded (*22, 23*).

In addition to regulating cellular processes including metabolism, transcription, cell cycle control, protein quality control, and protein waste removal, the proteasome has been shown to have several roles in DNA repair. Disruption of the mammalian ubiquitin−proteasome system with a conditionally mutant E1 ubiquitin ligase reduces nucleotide excision repair (NER), and treatment of cells with the proteasome inhibitor MG132 reduces recruitment of the xeroderma pigmentosum protein C (XPC) repair factor to sites of UV-induced DNA damage (*24*). Additionally, the carboxy terminus of Rad23 is thought to have a role in regulating NER function by binding to Rad4 (the yeast orthologue of XPC) and protecting Rad4 from degradation by the ubiquitin−proteasome system (*25–28*). Disruption of mHR23B, but not mHR23A, caused a reduction of XPC protein levels, and disruption of both mHR23A/B resulted in a further reduction of XPC levels (*26*). In yeast, Rad23 interacts with the proteasome through its amino-terminal ubiquitin-like domain, and deletion of this domain results in UV sensitivity (*29*). However, these interactions appear to be independent of each other as simultaneous expression of Rad23 amino- and carboxy-terminus mutants fully suppresses the UV sensitivity of a *rad23*Δ strain (*25*). In addition, interaction of human HR23 proteins with the human 3-methyladenine-DNA glycosylase (AAG) was shown to stimulate AAG activity with damaged DNA (*30*). Finally, Krogan et al. (*31*) have demonstrated that recruitment of the proteasome to a double-strand break in vivo depends on components of both the homologous recombination and the nonhomologous end-joining pathways and that this activity was dependent on Sem1, a subunit of the 19S proteasome. Taken together, the multiple ties between the DNA repair and the proteasome suggest that intimate associations between these pathways are required to modulate toxicity to damaging agents.

In contrast to the highly selective proteasome, the yeast vacuole (analogous to the mammalian lysosome) is an essential organelle responsible for bulk turnover of proteins by the nonselective process of autophagic uptake (*17, 32*). Autophagy is the most prominent mechanism of protein entry into the vacuole, whereby vesicles containing cargo such as membrane proteins, organelles, and fully folded protein complexes fuse with the vacuolar membrane. However, proteins can also enter the vacuole via endocytosis (e.g., extracellular proteins) and selective uptake (*33–35*). Biochemical studies with phenylmethanesulfonyl fluoride (PMSF), which prevents autophagic body formation as well as inactivation of many vacuolar serine proteases, suggest that the vacuole has a primary role in degrading long-lived proteins in yeast (*36*).

Here, we present data demonstrating that global protein degradation is significantly increased upon exposure to several classical DNA-damaging agents. We show that the observed increase in protein degradation for at least two of the agents is primarily dependent on proteasome function but not vacuolar function. However, inhibition of both proteasome and vacuolar function is required to suppress growth after acute exposure to MMS. Our data also suggest that the signal for accelerated protein degradation does not originate from DNA damage and that the damage-induced protein degradation response is not dependent on the environmental stress response (ESR) or endoplasmic reticulum-associated protein degradation (ERAD).

Experimental Procedures

***S. cerevisiae* Strains, Culture Media, and Materials.** *S. cerevisiae* strain BY4741 and its derivatives were purchased from Research Genetics (Carlsbad, CA). YPD media was prepared as 1% yeast extract/2% peptone/2% glucose. Complete synthetic media without methionine (CSM-Met) was prepared as described by the manufacturer (Qbiogene, Inc.). MMS, *t*-BuOOH, PMSF, L-methionine, and cycloheximide were purchased from Sigma, 4NQO was from Supelco, and MG132 was from Calbiochem.

Measurement of Protein Degradation. Protein degradation was measured using a protocol adapted from Lee et al. (*37*). Yeast cells were grown at 30 °C to an OD_{600} between 0.5 and 1.0 in CSM-Met supplemented with 20 μg/mL methionine, and the cultures were split, incubated with damaging agent (0.3% MMS, 5 mM *t*-BuOOH, or 2 μg/mL 4NQO) or untreated for 20 min at 30 °C, then harvested by centrifugation, and resuspended in CSM-Met at one-third the original volume. For UV-C (254 nm) treatment, cells were harvested by centrifugation and resuspended in CSM-Met at one-third the original volume, placed in a Petri dish, and exposed to 1000 J/M^2 UV-C. After treatment, the cells were labeled for 5 min with 100 μCi of [^{35}S]methionine, (1175 Ci/mmol) (Perkin-Elmer) per culture, washed twice with phosphate-buffered saline (PBS), and resuspended in CSM-Met containing 0.5 mg/mL methionine and 0.5 mg/mL cycloheximide. Aliquots were removed over a 3 h time course and mixed with 100% trichloroacetic acid (TCA) to give a final concentration of 10% TCA. After 1 h on ice, the samples were centrifuged and the TCA precipitable material (pellet) was washed once with distilled H_2O and solubilized in scintillation fluid (Fisher). The radioactivity was measured using a Beckman LS 6000IC scintillation counter. A consistent decrease in the level of [^{35}S]-labeling for the MMS-treated cultures was observed (results not shown). The percent degradation was calculated based on the initial level (time zero) of radioactivity incorporated into the TCA precipitable material for a culture. The percent degradation for each time point of a culture was calculated based on the radioactive counts for the TCA precipitable material for that time point divided by the TCA precipitable material for the zero time point multiplied by 100% (fraction of signal lost from initial signal). This value was then subtracted from 100% (total signal incorporated at time zero) to yield a percent degradation for the time point. Consequently, the zero time point corresponds to 0% degradation and subsequent time points correspond to a value greater than zero.

Protein degradation was measured in the presence of proteasomal or vacuolar inhibitors in the following way: Subsequent to the initial growth YML008C (*erg6*Δ), cultures were split, concentrated 3-fold in CSM-Met supplemented with methionine, and preincubated with 100 μM MG132 or 1 mM PMSF and vehicle [dimethyl sulfoxide (DMSO) or ethanol, respectively] for 90 min prior to treatment with damaging agent. [^{35}S]Methionine labeling and TCA precipitation were performed as above; however, the cells were only washed once with PBS following the labeling procedure and the distilled H_2O wash prior to solubilization was omitted. The inhibitors were present in the culture media for both the labeling and the chase periods.

Growth Inhibition Assays. A culture of *erg6*Δ cells was grown at 30 °C to an OD_{600} of about 0.03 in YPD media. To 20 mL cultures at this density we added vehicle, MG132, PMSF, or MG132 plus PMSF. After incubation at 30 °C for 90 min, the cultures were split and treated with 0.3% MMS or untreated for 20 min. Cells were harvested and resuspended in 10 mL of YPD containing inhibitors or vehicle. The OD_{600} of each culture was monitored over a 48 h time course using a Beckman-Coulter DU 640 spectrophotometer.

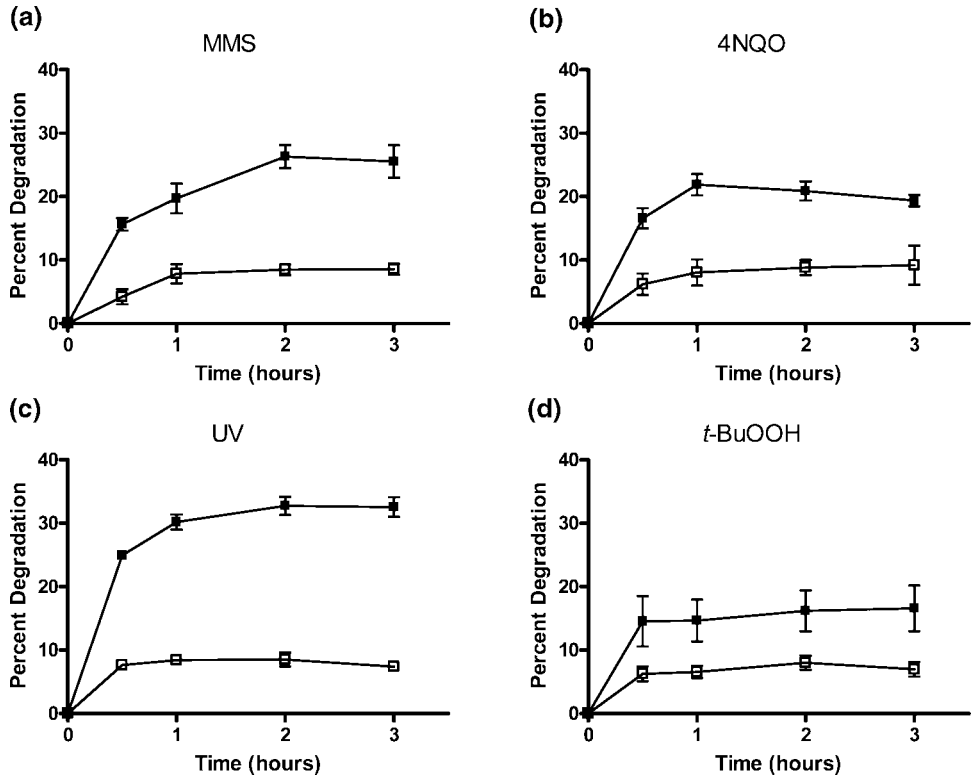

Figure 1. Protein degradation profiles for wild-type yeast with roughly equitoxic doses of classical DNA-damaging agents. Cultures were grown to midlog phase at 30 °C, exposed to damaging agent or not treated, pulse-labeled with 100 μCi [^{35}S]methionine, and chased with excess methionine and cycloheximide. Aliquots were removed and added to TCA at a final concentration of 10% over a 3 h time course. Cultures were exposed to the following damaging agents: (a) 0.3% MMS for 20 min (33% viability), (b) 2 μg/mL 4NQO for 20 min (22% viability), (c) 1000 J/m^2 UV (12% viability), and (d) 5 mM *t*-BuOOH for 20 min (56% viability). Data are plotted as the mean ± SE of four independent experiments. For cytotoxicity assays, cells were treated as above, diluted, and plated on YPD plates to measure colony formation. Percent viability for three independent assays is reported in parentheses above. Filled boxes, treated; open boxes, untreated.

Results

Acute Exposure to Classical DNA-Damaging Agents Results in Increased Protein Degradation. As whole genome studies had repeatedly indicated a role for the protein degradation machinery in modulating toxicity to classical DNA-damaging agents, we set out to determine whether the rate of global protein degradation changed upon exposure to such agents. Basal and agent-induced protein degradation was monitored as described in the Experimental Procedures by following the fate of pulse-labeled radioactive proteins. In short, our protocol consisted of a 20 min exposure to damaging agent, followed by 5 min of radioactive labeling and a 3 h chase period with cycloheximide during which the loss of radioactive signal for the cellular proteins was monitored (see Supporting Information, Figure S1). It is important to note that our assay monitors degradation of all cellular proteins, both damaged and undamaged. This methodology allowed adequate time for induction of the proteasome genes following exposure to damaging agent (*14*). The prevention of protein synthesis by addition of cycloheximide immediately following the radioactive pulse allows for a short, distinct radioactive pulse. The addition of cycloheximide prior to exposure of the damage agent would prevent expression of damage-induced genes. The addition of cycloheximide at a point in the procedure more distant from the labeling step would allow radioactive methionine to be recycled into newly synthesized proteins after the pulse period, effectively muddying the signal. Figure 1a shows the protein degradation profile for midlog phase wild-type yeast after a 20 min exposure to 0.3% MMS; this dose of MMS is moderately toxic, resulting in greater than 30% survival. For the MMS-treated cells, protein degradation was much more extensive than that observed in the untreated cells during a 3 h time course (Figure 1a). Similar protein degradation kinetics were observed in 4NQO-treated cells at a dose roughly equitoxic to that for MMS (Figure 1b). Exposure to 1000 J/m^2 UV resulted in even greater protein degradation with more than a 3-fold increase as compared to untreated cells (Figure 1c). Finally, exposure to a roughly equitoxic dose of *t*-BuOOH induced a significant but less dramatic increase in protein degradation (Figure 1d); nonetheless, degradation for the *t*-BuOOH-treated cultures was about 2-fold greater than that of the untreated cultures.

Damage-Induced Protein Degradation Is Dependent on Proteasome Function. To determine whether the observed increase in damage-induced protein degradation was dependent on proteasomal or vacuolar function, we measured protein degradation rates in the presence of proteasome or vacuolar inhibitors. For these experiments, degradation was measured in *erg6*Δ cells that have increased permeability, allowing entry of the proteasomal inhibitor MG132 into the cell. Cultures of *erg6*Δ cells were pretreated with inhibitor for 90 min prior to a 20 min exposure to the damaging agent as outlined in ref *36* and the Experimental Procedures. Basal and MMS-induced protein degradation for *erg6*Δ cells were both lower as compared to the wild-type cells, possibly due to a slower growth rate for *erg6*Δ mutants; nonetheless, exposure to DNA-damaging agents induced a 2-fold increase in protein degradation (Figure 2). Figure 2a shows the protein degradation profile for MMS-treated and untreated *erg6*Δ cells in the presence and absence of MG132. Inhibition of proteasomal function with MG132 reduced the MMS-induced protein degradation to a level almost

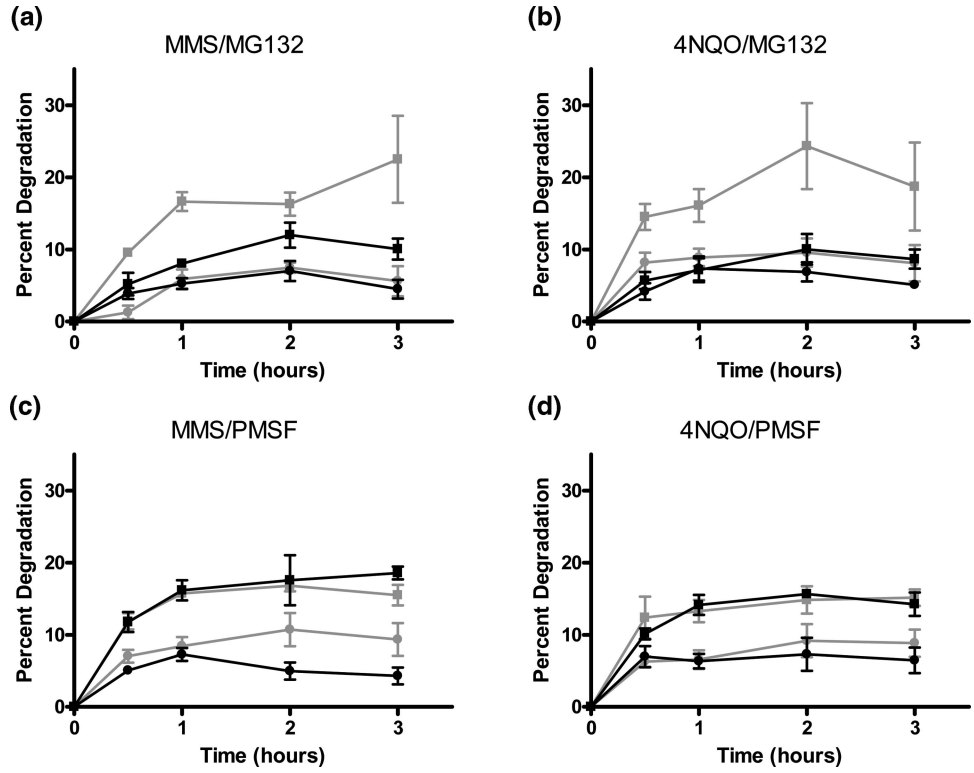

Figure 2. Protein degradation profiles for *erg6*Δ mutants after acute exposure to MMS or 4NQO in the presence and absence of proteasome and vacuolar inhibitors. Cultures were grown to midlog phase at 30 °C and pretreated with 100 μM MG132, 1 mM PMSF, or vehicle for 90 min and then treated and assayed as in Figure 1 in the presence or absence of inhibitors. Cultures were exposed to the following damaging agents for 20 min with chronic exposure to inhibitors: (a) 0.3% MMS and 100 μM MG132, (b) 8 μg/mL 4NQO and 100 μM MG132, (c) 0.3% MMS and 1 mM PMSF, and (d) 8 μg/mL 4NQO and 1 mM PMSF. Data are plotted as the means ± SE of four independent experiments. Black boxes, treated with inhibitor; black circles, untreated with inhibitor; gray boxes, treated, no inhibitor; and gray circles, untreated, no inhibitor.

as low as the untreated culture. Interestingly, MG132 did not reduce the basal level of protein degradation in untreated cells (Figure 2a). Very similar results were obtained for the 4NQO-treated cells in the presence and absence of MG132, except that in this case MG132 completely abolished the damage-induced increase in protein degradation (Figure 2b). It should be noted that no significant decrease in basal protein degradation was observed for this set of experiments with MG132 treatment alone (Figure 2b). This is in contrast to the results of Lee et al. (*36*) in that they observed a significant decrease in basal protein degradation for *erg6* cells with MG132 treatment. This discrepancy may be due to differences in uptake or metabolism of MG132 between strain backgrounds. Regardless, our results showing a reduction of damage-induced degradation with MG132 demonstrate that the inhibitor is active in our strain background. In contrast to proteasome inhibition by MG132, PMSF-mediated inhibition of vacuole function did not reduce damage-induced protein degradation following treatment with MMS or 4NQO (Figure 2c,d).

Proteasome and Vacuolar Function Are Required for Long-Term Growth after Acute MMS Exposure. Genomic phenotyping experiments support the idea that proper protein degradation function is required for cell growth upon chronic exposure to classical DNA-damaging agents (*15*). On the basis of the biochemical results presented above, we chose to determine whether an acute exposure to MMS with chronic exposure to proteasomal and/or vacuolar inhibitors would cause growth inhibition. Early log-phase *erg6*Δ culture was pretreated for 90 min with inhibitors or vehicle, exposed to 0.3% MMS or untreated for 20 min, and resuspended in fresh media containing the appropriate inhibitor cocktail to begin the time course. Figure 3 shows that chronic exposure to the proteasomal inhibitor MG132 or the vacuolar inhibitor PMSF inhibits growth slightly as compared to the untreated culture but that both cultures reach saturation 18 h into the time course. Chronic exposure to both inhibitors modestly, but significantly, inhibits growth, and these cultures do not reach saturation until 32 h into the time course. Treatment with MMS results in growth curves that are virtually indistinguishable for the no inhibitor and MG132- and PMSF-treated cultures, while the culture treated with MMS, MG132, and PMSF shows no growth for the entire duration of the time course (Figure 3). These results suggest that optimal proteasome and vacuole function are required for cell growth after acute exposure to alkylating agents.

Protein Degradation Profiles for Ubiquitin−Proteasome System and Vacuolar Mutants. We next investigated whether the protein degradation profiles in mutant strains compromised for the ubiquitin−proteasome system and vacuolar function are altered as compared to the wild-type basal and MMS-induced profiles. Because the proteasome and vacuole are essential for growth, activity cannot be completely abolished, such that truly null mutants are inviable. Nonetheless, we were able to test several mutants that have impaired proteasome or vacuole function, most of which are sensitive to classical DNA-damaging agents (*15*). Surprisingly, no significant change in the basal or MMS-induced protein degradation profile was observed for the ubiquitin−proteasome system mutants tested (*rpn4*Δ, *rpn10*Δ, *ubi4*Δ, and *ydj1*Δ mutants) (Figure 4a−d). These results were unanticipated. A reduction in MMS-induced protein degradation in *rpn4*Δ cells would be consistent with a role for Rpn4 in inducing proteasomal genes in an MMS-dependent manner; however, we did not observe a significant difference in the level of MMS-induced protein degradation for the *rpn4*Δ mutant as compared to wild type (Figure 4a). Rpn10 is a nonessential part

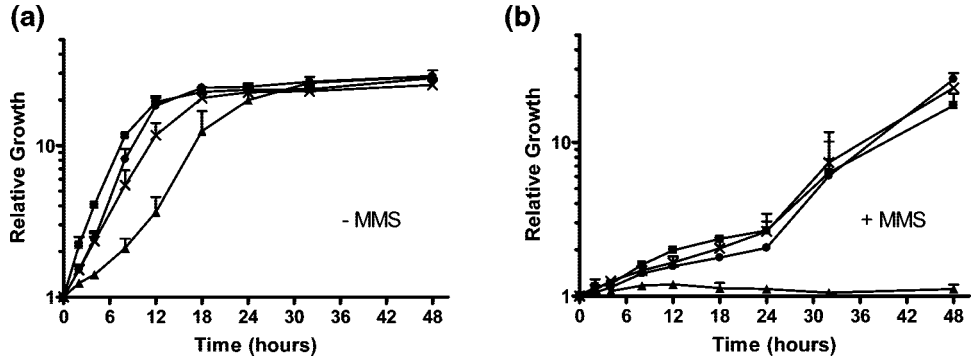

Figure 3. Growth inhibition assay for *erg6*Δ mutants after acute exposure to MMS in the presence and absence of proteasome and/or vacuolar inhibitors. A culture was grown to early log phase at 30 °C, divided into four equal volume cultures, and pretreated with 100 μM MG132, 1 mM PMSF, 100 μM MG132 and 1 mM PMSF or vehicle for 90 min. These cultures were then split and exposed to 0.3% MMS or untreated for 20 min, centrifuged at 3 kRPM for 3 min, and resuspended in fresh media containing appropriate inhibitors. Growth was monitored by measuring OD_{600} over a 48 h time course. (a) Untreated culture and (b) MMS treated. Data are plotted as the means ± SE of three independent experiments. Black boxes, no inhibitor; ×, MG132 treated; black circles, PMSF treated; and black triangles, MG132/PMSF treated.

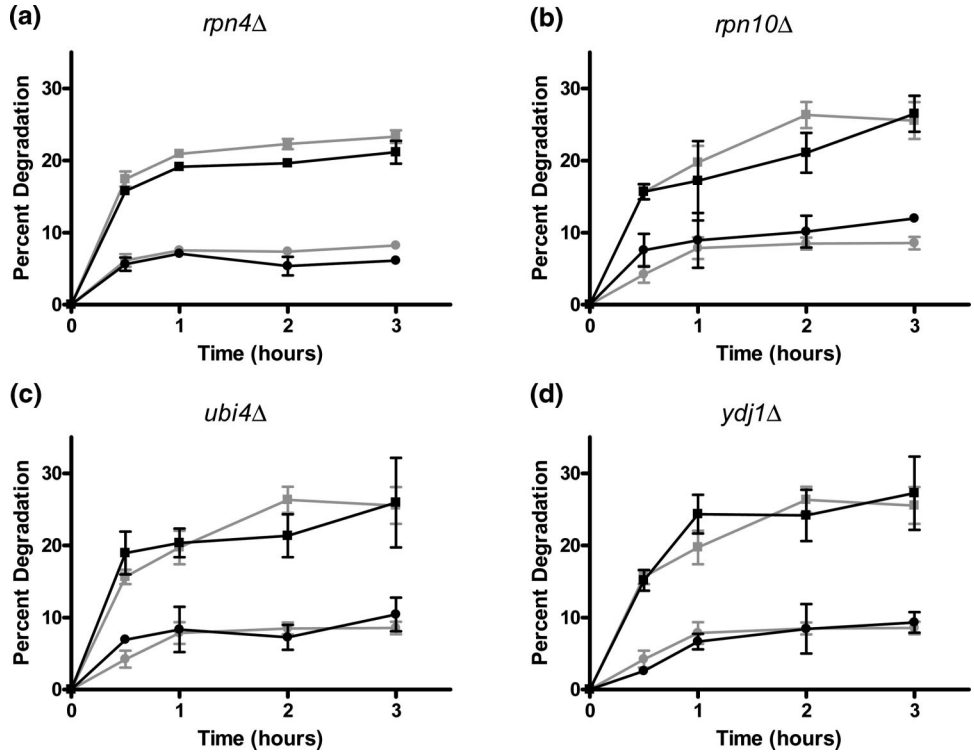

Figure 4. Protein degradation profiles for ubiquitin−proteasome system mutants after acute exposure to MMS. Protein degradation was assayed as in Figure 1. Data are plotted as the means ± SE of at least three independent experiments. Black boxes, MMS-treated mutant strain; black circles, untreated mutant strain; gray boxes, MMS-treated wild type; and gray circles, untreated wild type.

of the proteasome regulatory particle and is involved in substrate recognition for certain proteins (*38, 39*), and *RPN10* is transcriptionally induced following MMS treatment; despite this, *rpn10*Δ mutants are not sensitive to MMS (*12, 15, 16*) and MMS-induced protein degradation is unaffected (Figure 4b). The *UBI4* gene that encodes the polyubiquitin precursor to ubiquitin is also transcriptionally induced upon MMS exposure, but in this case, deletion of *UBI4* confers MMS sensitivity (*12, 15, 16*); the *ubi4*Δ strain was not compromised for MMS-induced protein degradation (Figure 4c). Because the *S. cerevisiae* genome contains four ubiquitin coding loci, *UBI4* is not essential for growth and is only required under conditions of stress (*40, 41*). It therefore seems likely that the level of ubiquitin in a *ubi4*Δ strain was sufficient to sustain the level of protein degradation induced by MMS exposure. Ydj1 is a protein chaperone required for selective degradation of certain normal and abnormal proteins. The level of protein degradation for *ydj1* mutants is reduced at elevated temperatures or in the presence of amino acid analogues, suggesting a role for Ydj1 in recognition of unfolded proteins (*37*). The *YDJ1* gene is transcriptionally induced following MMS treatment, and *ydj1*Δ mutants are sensitive to MMS, 4NQO, and UV irradiation. Nevertheless, *ydj1*Δ mutant cells were not compromised for either basal or MMS-induced protein degradation (Figure 4d). Because Rpn10 (*39*) and Ydj1 (*37*) are clearly selective in their substrates, it is formally possible that defects in MMS-induced protein degradation may be masked by assaying for degradation of the entire proteome.

The protein degradation profiles for mutants required for vacuolar function are displayed in Figure 5. Pep3 is a vacuolar peripheral membrane protein that mediates protein transport to the vacuole and promotes vesicular docking (*42, 43*) while Vam3 is required for vacuolar assembly and functions with Vam7 in vacuolar protein trafficking (*44, 45*). Both genes are

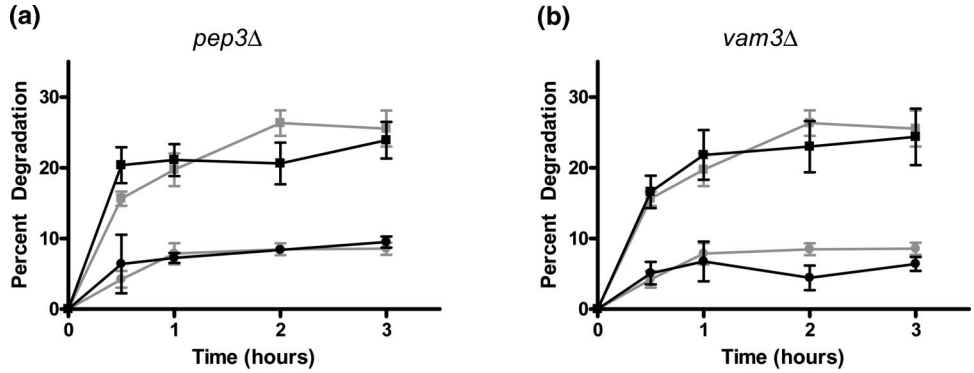

Figure 5. Protein degradation profiles for vacuolar function mutants after acute exposure to MMS. Protein degradation was assayed as in Figure 1. Data are plotted as the means ± SE of at least three independent experiments. Black boxes, MMS-treated mutant strain; black circles, untreated mutant strain; gray boxes, MMS-treated wild type; and gray circles, untreated wild type.

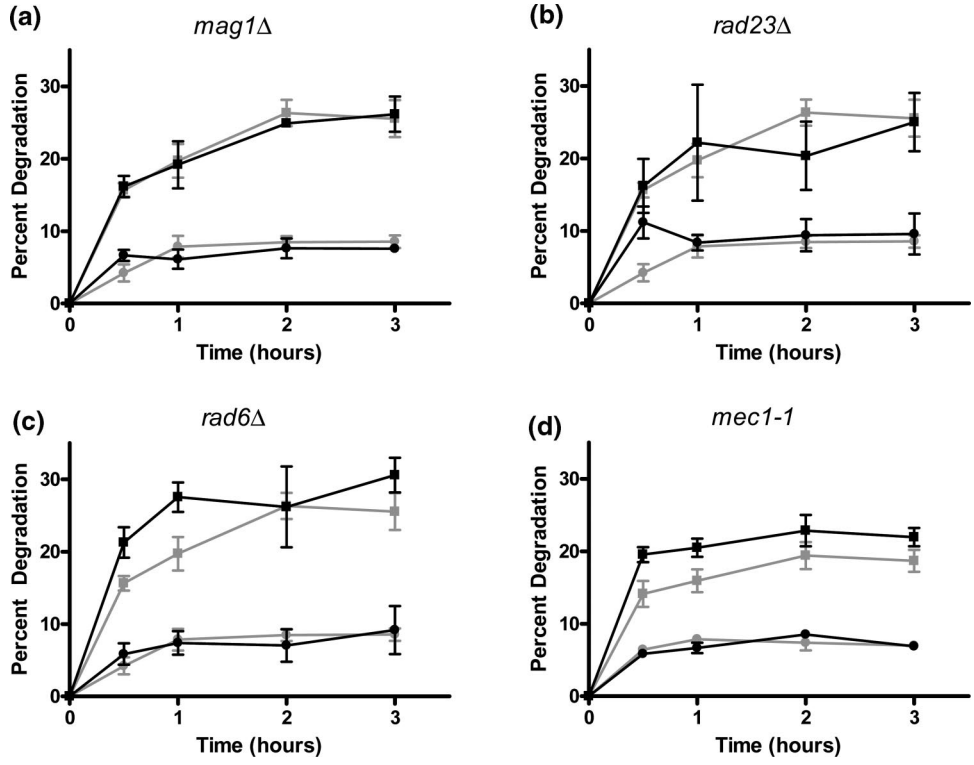

Figure 6. Protein degradation profiles for DNA repair mutants after acute exposure to MMS. Protein degradation was assayed as in Figure 1. Data are plotted as the means ± SE of at least three independent experiments. Black boxes, MMS-treated mutant strain; black circles, untreated mutant strain; gray boxes, MMS-treated wild type; and gray circles, untreated wild type.

transcriptionally induced upon MMS exposure, and deletion of either gene confers sensitivity to MMS, 4NQO, and *t*-BuOOH, with the *vam3*Δ mutants being sensitive to UV irradiation as well (*12, 15*). However, neither *pep3*Δ nor *vam3*Δ mutants displayed significantly altered basal or MMS-induced protein degradation (Figure 5a,b). This result is consistent with our results with PMSF, where vacuole inhibition did not show a clear effect on basal or damaged-induced protein degradation (Figure 2c,d).

The Signal for Increased Protein Degradation Does Not Appear To Originate from DNA Damage. We investigated whether the signal for increased protein degradation induced by DNA-damaging agents originates from DNA damage by determining protein degradation profiles for mutants involved in initiating the base excision repair (BER), NER, and postreplication repair (PRR) DNA repair pathways, as well as the Mec1 DNA damage-signaling pathway. Mag1 is the major 3-methyladenine DNA glycosylase in yeast (*46*). Following DNA alkylation damage, BER is initiated by Mag1-mediated removal of damaged bases (*47*). *MAG1* transcription is induced upon exposure to MMS, and *mag1* mutants are extremely MMS sensitive (*48*). The *MAG1* gene is coregulated with several proteasome genes as well as other genes involved in protein degradation and DNA repair, and all are regulated in a Rpn4-dependent manner, suggesting a coordinated DNA repair and protein degradation response to alkylation damage (*14*). 3-Methyladenine DNA lesions block replication by inhibiting DNA polymerases (*49, 50*). This inhibition of DNA synthesis is capable of signaling cell cycle arrest (*51, 52*), and it seemed plausible that a protein degradation response could also be signaled by the replication block. If DNA polymerase stalling at 3-methyladenine lesions were to elicit a protein degradation response, an increase in protein degradation would be expected in a *mag1* mutant due to increased replication blockage (*52*). Figure 6a shows that a *mag1*Δ strain has a protein degradation profile that is remarkably similar to the isogenic wild type, suggesting that Mag1 DNA damage substrates do not elicit the protein degradation response.

Similar results were obtained with *rad23*Δ and *rad6*Δ mutants (Figure 6b,c). The Rad23/Rad4 (NEF2) heterodimer preferentially binds damaged DNA, initiating NER (*53*). NEF2 is required for the incision step of NER, and NEF2 is suspected to play a role in recruiting other NER repair proteins to the site of damage (*54, 55*). Binding of the Rad6/Rad18 complex to damaged DNA initiates two branches of the PRR pathway (*56, 57*). The error-free lesion bypass branch is initiated with the interaction of the Rad6/Rad18 complex with Rad5 and recruitment of PCNA and Polδ to the damaged site (*57*). The error-prone lesion bypass branch is initiated after dimerization of the Rad6/Rad18 complex and recruitment of the error-prone lesion bypass polymerase Polζ. In the absence of the Rad6/Rad18 complex, another branch of the PRR pathway is initiated, which is also error-prone and is initiated by Rad5 recruitment of Polζ to the damaged site (*57*). The observation that the *rad23*Δ and *rad6*Δ strains, both of which are deficient in the damage recognition steps of NER and PRR, respectively, have a basal and MMS-induced protein degradation profile no different from that of wild type (Figure 6b,c) suggests that the DNA damage that is normally repaired by these repair pathways does not provide the signal for the damage-induced protein degradation response. It should also be noted that the DNA repair mutant results also suggest that repair intermediates such as single-stranded or nicked DNA do not signal the damage-induced protein degradation response. Additionally, the protein degradation profiles for *rad23*Δ mutants with and with out UV irradiation display no statistical deviation from the protein degradation profiles of similarly treated wild-type cultures (data not shown).

Mec1 is the yeast homologue of the human ATR protein, a phosphoinositol-3 kinase-related protein (*58–60*). Mec1 activates the checkpoint kinases Chk1 and Rad53 (CHK1/CHK2 homologues) (*61–63*), and signal transduction from certain kinds of DNA damage (e.g., strand breaks) involves the Mec1 pathway (reviewed in ref *64*). Figure 6d shows protein degradation for the *mec1-1* mutant (DLY285, MAT*a mec1-1::HIS3 ura3 leu2 trp1 his3*) (*52*) vs its isogenic wild-type strain (TWY397, MAT*a ura3 his7 leu2 trp1*) (*60*). Interestingly, the MMS-induced protein degradation for the *mec1-1* mutant is slightly greater than wild type for the duration of the experiment. *mec1* mutants are hypersensitive to most DNA-damaging agents as a consequence of being defective in cell cycle arrest and defective in initiating the ESR in response to DNA damage (*11, 59, 65*). The ESR results in the induction of genes involved in reactive oxygen species detoxification, carbohydrate metabolism, and various aspects of protein folding and degradation including chaperones, vacuolar proteins, and ubiquitin-conjugating enzymes (*11*). Increased damage-induced protein degradation in the *mec1-1* mutant suggests the following: (i) Mec1-mediated DNA damage signaling is not required to induce the observed increase in protein degradation after treatment with MMS, and (ii) the ESR is not required for the observed increase in protein degradation after damage.

Discussion

Our results show that global protein degradation is increased upon exposure to a number of classical DNA-damaging agents. We chose damaging agent concentrations that were roughly equitoxic to compare protein degradation responses. While the most toxic treatment (UV, 12% survival) resulted in the highest level of damage-induced protein degradation and the least toxic treatment (*t*-BuOOH, 56% survival) resulted in the lowest level of damage-induced protein degradation, a consistent correlation between the toxicity and the level of damage-induced protein degradation was not observed for these agents during this assay. As an example, MMS-induced protein degradation in the *mag1*Δ strain that is extremely sensitive to MMS-induced toxicity (*15*) did not differ from that in the wild-type strain that is relatively MMS-resistant (as measured over a 60 h time course). This was found to be true for at least one other strain background (unpublished observations). This suggests that the protein degradation profiles that we observe over the duration of our protein degradation assay truly represent intracellular protein degradation and are not simply the result of cellular breakdown following cell death.

To monitor protein degradation, several different approaches were explored as we developed our assay. Preliminary assays included radioactive labeling prior to MMS exposure. This included a cold chase immediately following the labeling step with simultaneous exposure to damaging agent. Cycloheximide was added during the exposure period or immediately following it. For these assays, the protein degradation profiles were indistinguishable for MMS-treated and untreated cultures (unpublished observations). The addition of cycloheximide during the exposure period would prevent expression of the damage-induced proteasome genes; therefore, a MMS-induced protein degradation response would not be expected. In addition, treatment in this manner would suppress any effects of protein damage due to incorporation of alkylated amino acids during protein synthesis. The addition of cycloheximide after exposure to MMS (with radioactive labeling prior to MMS exposure) resulted in poor quality data and similar protein degradation profiles for MMS-treated and untreated cultures (unpublished observations). Poor-quality data are believed to be a result of recycling of radioactive amino acids from degraded proteins back into newly synthesized proteins during the MMS treatment. This procedure would not result in a short, distinct radioactive pulse in the cellular proteins, which would produce a signal that could easily be monitored over the time course. Instead, this procedure would result in a prolonged, leaky incorporation of radioactivity in the cellular proteins, causing a dilution of the radioactive pulse and impaired measurement of protein degradation. For these reasons, we chose to perform our assay to measure protein degradation as outlined in the Experimental Procedures and diagrammed in Figure S1. Essentially, for the untreated cultures, our assay procedure and results are identical to those of Lee et al. (*37*).

Our protein degradation assay has been designed to measure the decay of protein-incorporated radioactivity for the entire yeast proteome for cultures that have or have not been exposed to classical DNA-damaging agents. As discussed below, no single mutant of the ubiquitin−proteasome system or the vacuolar degradation pathway showed a protein degradation profile that was significantly altered from that of wild type, most likely due to the redundancy of these pathways. However, in the presence of proteasome inhibitor MG132, we observed a significant decrease in the level of damage-induced protein degradation. No alteration in the level of damage-induced protein degradation was observed with the addition of vacuolar inhibitor PMSF to the media. These observations strongly suggest that the damage-induced protein degradation response is mediated primarily by the ubiquitin−proteasome system. Because proteasome genes are upregulated following exposure to damaging agents (*14*), it is entirely possible that the observed increase in protein degradation following exposure to damaging agent is due to an increase in protein turnover in general, that is, both damaged and undamaged proteins, not specifically degradation

of damaged proteins. Because there is a time span of about 40 min between the addition of damaging agent and the addition of cycloheximide, time is provided for protein synthesis with a potentially damaged amino acid pool. Therefore, incorporation of damaged amino acids into newly synthesized proteins may also contribute to the observed increase in protein degradation for cultures exposed to damaging agents.

For good reason, the majority of the current literature concerning the DNA-damaging agents used in this study focuses on their role in damaging the genome, with scant regard for damage to the many other intracellular macromolecules. However, reports demonstrating oxidative damage to proteins (*66–68*) and lipids (*69–71*), as well as alkylation damage to proteins, can be found in the literature (*72–74*). Lawley and Thatcher found that simple alkylating agents (dimethyl sulfate and MMS) preferentially methylated proteins over nucleic acids in mammalian cell cultures and that the rate of protein alkylation approximated the rate of hydrolysis (*75*). Whole animal studies have identified conjugates of *S*-methylcysteine in rat urine after administration of MMS (*76*) and have shown that subsequent to administration of dimethylnitrosamine, the level of methyl histidine in liver is elevated, but that six days after treatment, protein containing methyl histidine had largely disappeared (*77*). Taken together, these results support the notion that simple alkylating agents directly alkylate cellular proteins and that alkylated proteins are degraded in vivo.

Inhibition of both the proteasome and the vacuole is required to exacerbate MMS-induced inhibition of cell growth. MG132 is a reversible proteasome inhibitor that inhibits chymotrypsin-like and peptidylglutamyl peptide-hydrolyzing activity (*78*). For permeable yeast cells (*erg6* mutants), MG132 has been shown to decrease short-lived protein degradation by about 70% at the dose used in this study while PMSF decreases degradation of long-lived proteins by about 50% at the concentration used here (*36*). While the biochemical assay demonstrated that damage-induced short-term purging of proteins was performed mainly by the proteasome for at least two of the agents (MMS and 4NQO), our growth inhibition result suggests that subsequent to acute MMS exposure optimal vacuolar function is required to sustain growth when proteasome activity is reduced. This result is quite interesting in that it suggests a redundancy between the proteasome and the vacuole for purging the cell of alkylation-damaged proteins over the time course of the assay, even though vacuolar contributions to damage-induced protein degradation were not identified immediately following exposure to damaging agents.

The pathway controlling damage-induced protein degradation remains an enigma. Rpn4 controls transcription of almost all proteasome genes as well as many genes involved in ERAD, and *rpn4*Δ strains are ERAD-deficient (*79*). The fact that damage-induced protein degradation for *rpn4*Δ is not significantly decreased as compared to wild type suggests that MMS-induced protein degradation results from an alternative and possibly redundant pathway. It is plausible that the unfolded protein response pathway could mediate MMS-induced protein degradation; however, this seems unlikely in that deletion of the genes encoding transcriptional regulators of this pathway (Hac1 and Ire1) results in little or no MMS sensitivity (*15*). Furthermore, the transcriptional levels of *hac1* and *ire1* show little or no change following exposure to MMS (*12*). Additionally, Lee et al. (*37*) have shown that wild-type cells grown at elevated temperatures or in the presence of amino acid analogues have increased levels of protein degradation that increase to a level that is remarkably similar to what we see with the addition of damaging agents. This response is abolished for *ydj1* mutants assayed under the same conditions, suggesting that Ydj1 is involved in surveillance of abnormally synthesized proteins (*37*). The fact that the protein degradation profile for basal and MMS-induced protein degradation of *ydj1*Δ mutants was not altered as compared to wild type further suggests that it is unlikely that a major source of the damage-induced protein degradation is from aberrantly synthesized proteins. In addition, it has been repeatedly demonstrated that protein synthesis is downregulated upon exposure to MMS (*11, 12, 14, 80*).

Several groups have reported the involvement of the proteasome in disparate DNA repair pathways. Because of these links and the discovery of the coregulation of protein degradation genes and DNA repair genes upon MMS exposure, we chose to see if defects in DNA repair had an effect on protein degradation profiles. Mutants of the BER, NER, and PRR DNA repair pathways showed no deviation from wild type for basal or MMS-induced protein degradation. These data suggest that this cellular response to classical DNA-damaging agents is not dependent on signaling from intermediates in these DNA repair pathways. Furthermore, *mec1-1* mutants were not defective in damage-induced protein degradation. Because Mec1 transduces a variety of DNA damage signals, it seems unlikely that the signal to increase protein degradation in response to DNA-damaging agents emanates from damaged DNA. Altogether, our results suggest that damage-induced protein degradation is not regulated by DNA damage response mediators. These results beg the question, "What signals this increase in protein degradation?" Many of the proteasome genes are transcriptionally activated by Rpn4, a proteasome substrate known to have an extremely short half-life of about 2 min and known to regulate proteasome expression by a negative feedback circuit (*81*). Therefore, a reasonable answer to this question may be that a large increase of damaged proteins could overwhelm proteasome activity, leading to a transient increase in Rpn4 abundance, in turn rendering Rpn4 available to bind to the proteasome-associated control elements (PACE) upstream of proteasome genes (*82*), thus driving proteasome expression. This idea is quite tantalizing in that Rpn4 activates the *MAG1* gene through its upstream PACE sequence (MAG1-URS2) (*14, 83, 84*), raising the possibility that *MAG1* is induced as a result of increased protein degradation overwhelming proteasome activity, further linking the proteasome and DNA repair. However, the fact that the MMS-induced protein degradation in *rpn4*Δ mutants is not significantly reduced suggests another factor could drive proteasome expression or that the level of proteasome present in the cell is sufficient to clear the damaged proteins from the cell. Should the later point be true, then the observed increase in proteasome expression upon exposure to DNA-damaging agents (*14*) may be indicative of back-up activity. All together, our data suggest that multiple redundant pathways may be responsible for regulating the damage-induced protein degradation response.

The results presented here are important to consider when assessing the effects of chemotherapeutic cocktails containing alkylating agents and proteasome inhibitors. Like MMS, the classical alkylating chemotherapeutics, melphalan and cyclophosphamide, alkylate their substrates via a S_N2 mechanism, supporting the use of MMS as a model alkylating agent to study the putative effects of chemotherapeutics. One of the major modes of action for the proteasome inhibitor bortezomib in mediating anticancer affects is via apoptosis; however, its anticancer activity is not fully defined (*3, 4, 8*). Bortezomib is thought to affect many cell functions including cell adhesion,

angiogenesis, and DNA repair (*8, 85, 86*). Additionally, bortezomib suppresses the expression of growth factor genes while inducing expression of proapoptotic genes (*87*). Both bortezomib and MG132 inhibit the chymotrypsin-like proteolytic activity of the proteasome (*88, 89*). Our data are consistent with the idea that an increase in the intracellular concentration of damaged proteins, resulting from protein alkylation coupled with inhibition of the mechanism to purge the damaged proteins, results in a growth defect. Consideration of the combined effects of alkylating agents and proteasome inhibitors on cell fate as outlined in this study will aid in the rational design of therapeutic strategies to improve anticancer effects.

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Supporting Information Available: Protein degradation assay time course and growth inhibition time course. This material is available free of charge via the Internet at http://pubs.acs.org.

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