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Evidence That Inhibition of p44/42 Mitogen-activated Protein Kinase Signaling Is a Factor in Proteasome Inhibitor-mediated Apoptosis*

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The proteasome is emerging as a target for cancer therapy because small molecule inhibitors of its catalytic activity induce apoptosis in both *in vitro* and *in vivo* models of human malignancies and are proving to have efficacy in early clinical trials. To further elucidate the mechanism of action of these inhibitors, their impact on signaling through the p44/42 mitogen-activated protein kinase (MAPK) pathway was studied. Proteasome inhibition with either carbobenzoxy-leucyl-leucyl-phenylalaninal or lactacystin led to a loss of dually phosphorylated, activated p44/42 MAPK in A1N4-*myc* human mammary and MDA-MB-231 breast carcinoma cells in a dose- and time-dependent fashion. This correlated with an induction of the dual specificity MAPK phosphatases (MKP)-1 and -2, and blockade of MKP induction using either actinomycin D or Ro-31-8220 significantly decreased loss of activated p44/42 MAPK. Inhibition of p44/42 MAPK signaling by use of the MAPK kinase inhibitors PD 98059 or U0126, or by use of a dominant negative MAPK construct, enhanced proteasome inhibitor-mediated apoptosis. Conversely, activation of MAPK by epidermal growth factor, or use of a mutant MAPK resistant to MKP-mediated dephosphorylation, inhibited apoptosis. These studies support a role for inactivation of signaling through the p44/42 MAPK pathway in proteasome inhibitor-mediated apoptosis.

Eukaryotic intracellular protein degradation occurs predominantly through the ubiquitin (Ub)[1]-proteasome pathway, composed of the Ub-conjugating system and the 26 S proteasome (reviewed in Refs. 1–5). The former labels proteins with ubiquitin chains in preparation for recognition by the 26 S proteasome, whereas the latter contains the 20 S multicatalytic proteinase complex (1–5), with its proteolytically active subunits (reviewed in Ref. 6). Ub-proteasome pathway activity is essential for such fundamental cell functions as the timely degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (reviewed in Ref. 7), processing and degradation of transcription factors, short-lived regulatory proteins (1–5), antigen processing (reviewed in Ref. 8), and angiogenesis (9). A role for the proteasome in programmed cell death was uncovered using small molecular weight, cell-permeable inhibitors, which induce apoptosis in a variety of tumor-derived cell lines (reviewed in Refs. 10–12). In some cases the apoptotic program is preferentially activated in transformed cells, with relative sparing of nontransformed cells (13–15). Proteasome inhibitors have induced apoptosis and tumor growth delay in xenograft models of human malignancies (13, 16–18). Most recently, proteasome inhibitors have entered clinical trials, and preliminary evidence of significant anti-tumor activity has been reported (19).

It has been hypothesized that proteasomes act at a pre-mitochondrial, pre-caspase stage of apoptosis in some systems (10–12, 20). Given the importance of the proteasome to normal cellular homeostasis, however, it is likely that inhibitors induce programmed cell death by affecting many apoptosis-associated pathways. Indeed, evidence has been presented that, at least in some systems, has implicated roles for p53 accumulation (see, for example, Ref. 21), activation of c-Jun N-terminal kinase (22), and accumulation of $p27^{Kip1}$ (see, for example, Ref. 23). Investigations have also supported the importance of blockade of nuclear translocation of NF-κB (see, for example, Refs. 24 and 25), inhibition of degradation of pro-apoptotic Bid (26), and activation of the Fas-Fas ligand signaling system (27) in proteasome inhibitor-mediated apoptosis (reviewed more fully in Refs. 10–12).

Some of the more ubiquitous cellular signaling cascades are the mitogen-activated protein kinase (MAPK) pathways, including the p44/42 MAPK, also called the extracellular signal-related kinases, or ERK-1/2. These pathways play important roles in a variety of processes, including cell development, differentiation, inflammation, and apoptosis (reviewed in Refs. 28–30). A possible role for the proteasome in ERK-1/2 signaling has been suggested by studies with the peptidyl aldehyde inhibitor *N*-acetyl-Leu-Leu-norleucinal (ALLN), that targets both calpains and the proteasome (31–33), and induces nuclear accumulation of p44/42 MAPK in CCL39 Chinese hamster lung fibroblasts (34). The cysteine proteinase inhibitors calpeptin and E64D have been reported to inactivate MAPK function in Balb/c 3T3 cells by inducing MAPK phosphatase (MKP)-1, but ALLN did not exhibit a similar ability in these cells (35). In contrast, other studies in CCL39 cells reported that ALLN does

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[1] The abbreviations used are: Ub, ubiquitin; EGF, epidermal growth factor; ERK, extracellular signal-related kinase; HRP, horseradish peroxidase; LCCC TCF, Lineberger Comprehensive Cancer Center Tissue Culture Facility; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MKP, mitogen-activated protein kinase phosphatase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PP1, phosphoprotein phosphatase 1; PP2A, phosphoprotein phosphatase 2A; Z-LLF-CHO, carbobenzoxy-leucyl-leucyl-phenylalaninal; Z-LLF-OH, carbobenzoxy-leucyl-leucyl-phenylalaninol. Standard three-letter abbreviations are used in reference to amino acids.

indeed induce MKP-1 (36), but its impact on p44/42 MAPK was not investigated. Recent work with the proteasome inhibitor PS-341 (16) suggested that phosphorylation of p44/42 MAPK was decreased (37), but the impact of proteasome-specific inhibitors on the activation status of ERK-1/2 has not been well characterized. Furthermore, a role for this pathway in the induction of programmed cell death by proteasome inhibitors has not been described, and we therefore sought to investigate this possibility further.

In the current report, we present data demonstrating that inhibition of the proteasome in breast epithelial and breast carcinoma cells resulted in inhibition of signaling through ERK-1/2 by a decrease in the abundance of the active, dually phosphorylated kinases. This decrease was accompanied by the induction of MKP-1 and -2, and both occurred in a time- and concentration-dependent fashion. Blockade of MKP induction significantly inhibited MAPK dephosphorylation, suggesting that the two were closely linked. Inhibition of p44/42 MAPK signaling using both pharmacologic and dominant negative mutant constructs enhanced proteasome inhibitor-mediated apoptosis, whereas activation of MAPK by pharmacologic or genetic means decreased this programmed-cell death. These studies support a role for inhibition of MAPK signaling in the induction of programmed cell death by proteasome inhibitors.

EXPERIMENTAL PROCEDURES

Materials—The proteasome inhibitor carbobenzoxy-leucyl-leucyl-phenylalaninal (Z-LLF-CHO), synthesized by oxidation of the corresponding peptidyl alcohol, Z-LLF-OH, as previously described (38), was purified by crystallization from ethanol/water, whereas lactacystin was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Phosphatase inhibitors deltamethrin, nodularin, and okadaic acid were from Calbiochem-Novabiochem Corp., whereas sodium orthovanadate was from Sigma. Actinomycin D and phenylmethylsulfonyl fluoride (PMSF) were from Fisher Scientific (Fair Lawn, NJ), whereas Ro-31-8220 was from Calbiochem-Novabiochem Corp., as was the MEK inhibitor PD 98059, and U0126 was from Promega Corp. (Madison, WI). Stock solutions and serial dilutions were prepared in 100% ethanol (Z-LLF-OH, Z-LLF-CHO, and PMSF; Mallinckrodt Baker, Inc., Paris, KY), 100% methanol (actinomycin D; Fisher Scientific), phosphate-buffered saline (sodium orthovanadate (PBS); 1× PBS, 9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4; obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Facility (LCCC TCF)), or dimethyl sulfoxide (Fisher Scientific) for all others, and stored at −20 °C. These reagents were used at concentrations indicated in the text, with a final vehicle concentration that did not exceed 0.5% (v/v). All other chemicals, unless otherwise indicated, were obtained from Fisher Scientific.

Cell Lines and Cell Culture—A1N4-*myc* cells (39), the generous gift of Dr. R. B. Dickson (Georgetown University, Washington, DC), were grown in Richter's modified Eagle's medium with insulin, gentamicin, and 20 mM Hepes (LCCC TCF), and further supplemented with human recombinant epidermal growth factor (EGF) at 10 ng/ml unless otherwise indicated, 9% fetal bovine serum, penicillin G sodium at 100 units/ml, streptomycin sulfate at 100 μg/ml (these reagents from Invitrogen), and hydrocortisone (39) (Sigma). MDA-MB-231 (40) and BT-474 (41) human breast carcinoma cells (LCCC TCF) were grown in Richter's modified Eagle's medium as above except without EGF and hydrocortisone. All cells were propagated in incubators providing a humidified atmosphere with 5% CO_2 in air.

Evaluation of p44/42 MAPK Activation Status—Adherent cells were detached by exposure to trypsin (Invitrogen), counted using a hemacytometer (Hausser Scientific, Horsham, PA), and 2×10^6 cells were seeded onto 60-mm Falcon 3002 tissue culture plates (BD PharMingen Labware, Lincoln Park, NJ) in complete medium. These cells were allowed to reattach overnight and subjected to conditions described in the text along with the addition of fresh medium. At the completion of an experiment, all plates were placed on ice, washed with 5 ml of ice-cold PBS, and collected by scraping into eukaryotic lysis buffer (10 mM Tris-hydrochloride (HCl), pH 7.4, 2% sodium dodecyl sulfate (SDS), 5 mM EDTA, 100 μg/ml PMSF, 1 nM deltamethrin, 180 nM nodularin, and 100 μM sodium orthovanadate). Lysates were immediately denatured by heating to 95–100 °C for 10 min, and after the removal of aliquots for protein assays, samples were reduced by heating for another 5 min at 95–100 °C in 1× SDS sample buffer (31.25 mM Tris-HCl, pH 6.8, 2% SDS, 5% mercaptoethanol, 10% sucrose, and 0.005% bromphenol blue). Relative protein concentrations of each sample were determined using the BCA protein assay kit (Pierce).

Equivalent protein amounts were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the Broad Range Biotinylated Protein Marker set (New England Biolabs, Beverly, MA) and/or the Low Range Prestained SDS-PAGE Standards (Bio-Rad) using the Mini-PROTEAN II electrophoresis system (Bio-Rad). A discontinuous buffer system essentially according to Laemmli was used (42), and separated proteins were then electrophoretically transferred to BA85 Protran nitrocellulose filters (Schleicher & Schuell) using a Mini-PROTEAN II blotting system (Bio-Rad). These were blocked with a solution of nonfat dry milk (Nestle USA, Inc., Solon, OH) in 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.10% Tween 20, and exposed to the indicated antibodies diluted in this same solution. Horseradish peroxidase (HRP)-conjugated anti-rabbit (New England Biolabs or Bio-Rad), anti-mouse (Amersham Biosciences), or anti-rat (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) secondary antibodies were then used, with an anti-biotin HRP-conjugated antibody (New England Biolabs) as needed. Immunoreactive protein bands were detected using either the Phototope-HRP Western detection kit (New England Biolabs) or the ECL detection system (Amersham Biosciences), Kodak Biomax ML film, and a Kodak model M6B RP X-Omat film processor (Eastman Kodak Co.). For repeated analyses of the same filter, blots were stripped for 45 min using Western Re-Probe (Geno Technology, Inc., St. Louis, MO) following the manufacturer's specifications.

Activation status of the p44/42 MAPKs was determined using murine monoclonal antibodies recognizing active, dually phosphorylated p44/42 (Thr-202/Tyr-204), with rabbit p44/42 antibodies used as loading controls. The activation status of the mitogen-activated protein kinase kinase (MEK) was determined using rabbit polyclonal antibodies recognizing active, dually phosphorylated MEK-1/2 (Ser-217/Ser-221), with rabbit MEK-1/2 antibodies (all from Cell Signaling Technology, Inc., Beverly, MA) used as loading controls. Additional loading controls were provided by rat monoclonal antibodies 1B5 to HSC-70 and 9G10 to GRP-94 (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada), the former a generous gift of Dr. J. Spychala.

To quantify protein bands, autoradiographs were scanned with an Agfa Duoscan T2500 scanner (Agfa Corp., Ridgefield Park, NJ) into Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA), and densitometry was performed using NIH Image version 1.61. These procedures were performed by members of the laboratory who were blinded to the experimental conditions.

Evaluation of Phosphatase Expression—Western blotting performed as described above was used to evaluate the expression of the MKP-1 and -2 phosphatases. Primary antibodies used included rabbit polyclonal C-19 antibody to MKP-1 and S-18 to MKP-2 (Santa Cruz Biotechnology, Inc.). To inhibit phosphatases in intact cells, A1N4-*myc* and MDA-MB-231 lines were pre-incubated for 60 min with either sodium orthovanadate at 1 mM, okadaic acid at 30 nM, or both, and then subjected to conditions as described in the text and processed and analyzed as noted above. MKP-1 and -2 induction was inhibited by pre-incubation of cells for 60 min with either actinomycin D at 3.0 μg/ml, or Ro-31-8220 at 5.0 μM.

Apoptosis Assays—For apoptosis experiments cells were seeded onto either Falcon 3226 24-well plates (BD PharMingen Labware) at a density of 1×10^5 cells/well or Costar 3595 96-well plates (Corning Inc., Corning, NY) at a density of 1×10^4 cells/well in complete medium, unless indicated otherwise. Fresh medium was added containing the agents for which impact was being tested, and cells were analyzed for apoptosis using the Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals). Spectrophotometric data at a wavelength of 405 nm, with a reference of 490 nm, were acquired on a MAXline Vmax kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA). The enhancement of apoptosis induced by each condition was calculated in relation to parallel control cells which received vehicle alone, and tabulated in KaleidaGraph version 3.0.1 (Synergy Software, Reading, PA). Mean percentages and standard errors of the mean were then calculated and plotted in KaleidaGraph.

Cloning and Cell Line Preparation—The hyperactive, phosphatase-resistant allele of ERK2 (D319N) (43), as well as a dominant negative ERK2 allele (K52R) (44), both tagged with hemagglutinin, were generous gifts of Dr. C. Der (University of North Carolina, Chapel Hill, NC). These were cloned into the vector pLPCX (CLONTECH), where expression is driven by the human cytomegalovirus immediate early promoter, and verified by sequencing (DNA Sequencing Core Facility, Lineberger Comprehensive Cancer Center, Chapel Hill, NC). Stable cell

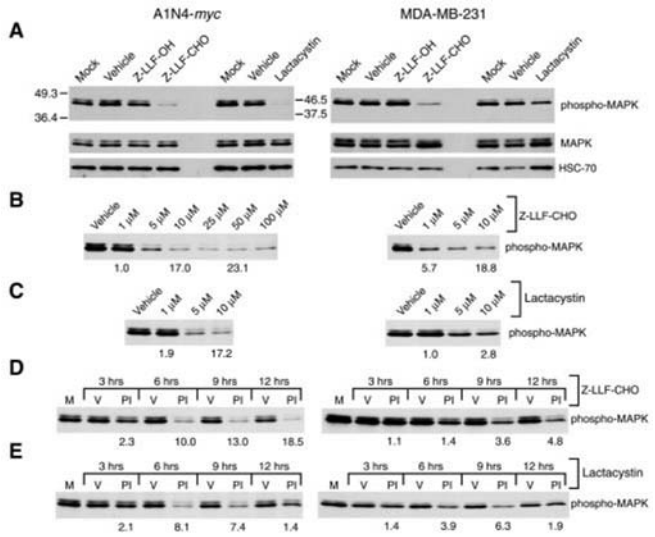

FIG. 1. **p44/42 MAPK in cells exposed to proteasome inhibitors.** *A*, A1N4-*myc* (*left panel*) or MDA-MB-231 cells (*right panel*) were mock-treated, exposed to vehicle, the inactive precursor Z-LLF-OH at 100 μM, the active peptidyl aldehyde proteasome inhibitor Z-LLF-CHO at 100 μM, or the structurally unrelated specific proteasome inhibitor lactacystin at 10 μM, for 6 (A1N4-*myc*) or 12 (MDA-MB-231) h. The impact on p44/42 MAPK signaling was evaluated by Western blotting using antibodies directed against the dually phosphorylated, active forms of ERK-1 and -2. Equal loading was confirmed by reprobing with antibodies that recognize ERK in a phosphorylation status-independent manner, and then with antibodies directed against HSC-70. The migration of pre-stained and biotinylated protein molecular size markers are indicated. All *panels* in this figure are representative results from one of two independent experiments. *B*, A1N4-*myc* or MDA-MB-231 cells were exposed to the indicated concentrations of Z-LLF-CHO for 6 or 12 h as above. The -fold decrease in phospho-MAPK abundance is shown in relation to vehicle-treated cells and is adjusted for loading of HSC-70 (data not shown). Higher concentrations of Z-LLF-CHO than those shown for MDA-MB-231 cells did not result in increased MAPK inactivation. *C*, A1N4-*myc* or MDA-MB-231 cells were exposed to the indicated concentrations of lactacystin for either 9 (A1N4-*myc*) or 12 (MDA-MB-231) h. *D* and *E*, A1N4-*myc* or MDA-MB-231 cells were exposed to 5 μM Z-LLF-CHO (*PI*) (*panel D*), 5 μM lactacystin (*panel E*), or vehicle (*V*) for 3, 6, 9, and 12 h prior to lysate preparation, whereas mock-treated cells (*M*) were fed with fresh medium for 5 min. The -fold decrease of phospho-MAPK levels is shown in relation to vehicle-treated cells harvested in parallel at each time point and is adjusted for loading of HSC-70.

lines were prepared in BT-474 cells by transfection using the GenePORTER™ reagent according to the manufacturer's specifications (Gene Therapy Systems, San Diego, CA), followed by selection in medium containing puromycin (Calbiochem-Novabiochem Corp.). Colonies were screened for mutant ERK expression by Western blotting, as described above, using a murine anti-hemagglutinin monoclonal antibody (F7; Santa Cruz Biotechnology, Inc.).

To prepare A1N4-*myc*- and MDA-MB-231-based cell lines, the indicated ERK clones were transfected into AmphoPack™-293 cells using the CalPhos™ mammalian transfection kit (both from CLONTECH) according to the manufacturer's specifications. Supernatants with retroviral particles were collected and used to infect the target cells in medium containing 8 μM Polybrene. Selection and screening of clones was performed as described above.

RESULTS

Proteasome Inhibitors Decrease Signaling through p44/42 MAPK—To determine the impact of proteasome inhibition on ERK-1/2 pathway signaling immortalized A1N4-*myc* cells, derived from benzo(a)pyrene-treated normal human mammary epithelium (39), and MDA-MB-231 human mammary carcinoma cells (40) were studied. Analysis of extracts after mock treatment or exposure to vehicle revealed significant levels of dually phosphorylated, active p44 and p42 MAPK in A1N4-*myc* (Fig. 1*A*, *left panel*) and MDA-MB-231 cells (Fig. 1*A*, *right panel*). When these cells were exposed to Z-LLF-OH, a precursor peptidyl alcohol with no activity as a proteasome inhibitor (38, 45), phospho-MAPK levels were unchanged. Treatment with the active peptidyl aldehyde proteasome inhibitor Z-LLF-CHO, however, resulted in decreased levels of phospho-ERK-1 and -2, which were not because of a loss of p44/42 protein. Total MAPK levels were themselves unchanged, as determined by evaluation for an unrelated protein, the heat shock cognate protein HSC-70. Many of the proteasome inhibitors previously used in studies of the p44/42 MAPK pathway (34, 35) have been peptides that also inhibit calpains, and Z-LLF-CHO falls into this category as well (31–33). Because more specific calpain inhibitors have also been reported to induce inhibition of MAPK function (35), we evaluated the impact of the specific proteasome inhibitor lactacystin (46). This agent also induced a loss of dually phosphorylated p44 and p42 MAPK (Fig. 1*A*), demonstrating that in these cells proteasome inhibition was by itself sufficient to reduce signaling through this pathway.

The effect of proteasome inhibitors on p44/42 MAPK signaling was tested further in concentration and time dependence studies. A1N4-*myc* (Fig. 1*B*, *left panel*) and MDA-MB-231 cells (Fig. 1*B*, *right panel*) exposed to Z-LLF-CHO both exhibited decreased phospho-ERK-1/2 levels in a manner that was dependent on the proteasome inhibitor concentration. Exposure to lactacystin also resulted in a concentration-dependent decrease in phosphorylated p44 and p42 MAPK (Fig. 1*C*, *left* and *right panels*). Time-course experiments revealed that A1N4-*myc* cells were especially sensitive in that phospho-MAPK levels decreased as early as 3 h after addition of either Z-LLF-CHO (Fig. 1*D*, *left panel*) or lactacystin (Fig. 1*E*, *left panel*), and decreased further with prolonged incubation in a time-dependent fashion. MDA-MB-231 cells showed a similar time dependence, although loss of active MAPK was somewhat delayed, and did not begin until 6 h after exposure to Z-LLF-CHO (Fig. 1*D*, *right panel*) or lactacystin (Fig. 1*E*, *right panel*). Inactivation of MAPK signaling was more transient with lactacystin for both A1N4-*myc* and MDA-MB-231 cells, however, in that phospho-ERK-1/2 levels reached their nadir at ~9 h and then began to return to baseline at 12 h. Because lactacystin is a reactive compound with a short half-life (46), the possibility was considered that the decline in MAPK inactivation after 9 h was because of the disappearance of the active proteasome inhibitor. When additional lactacystin was added to cell cultures after 6 h of incubation, phospho-p44/42 MAPK levels continued to decrease at 9 and 12 h (data not shown), further supporting the link between proteasome inhibition and MAPK inactivation.

Decreased ERK-1/2 Signaling Is Caused by a Phosphatase—Phosphorylation of p44/42 MAPK is catalyzed by the mitogen-activated protein kinase kinase MEK (29, 30), so the possibility was considered that decreased phospho-ERK-1/2 levels were because of decreased MEK activity. When A1N4-*myc* cells exposed to Z-LLF-CHO in time-course experiments were analyzed for their content of active phospho-MEK by Western blotting, however, no significant changes were noted (Fig. 2*A*), and indeed phospho-MEK levels seemed better preserved in the inhibitor-treated cells. A similar pattern was noted for lactacystin-treated A1N4-*myc* cells (data not shown), suggesting that under these conditions MEK activity was not significantly decreased, and therefore likely did not contribute to decreased phospho-ERK-1/2 levels. Another mechanism used by cells to control MAPK activity is through the action of protein phosphatases, including both phosphoprotein phosphatases 2A (PP2A) and 1 (PP1), as well as dual specificity protein phosphatases (reviewed in Refs. 47 and 48). To determine whether any of these phosphatases might be involved, cells were preincubated either with okadaic acid at concentra-

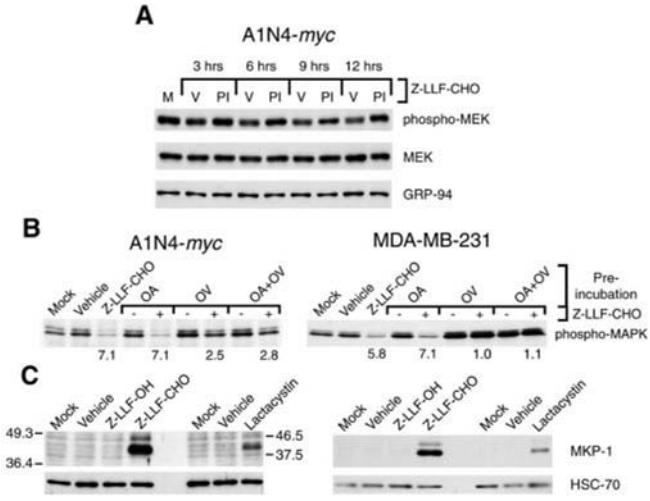

FIG. 2. **MAPK inactivation and cellular phosphatases.** *A*, A1N4-*myc* cells were exposed to 5 μM Z-LLF-CHO (*PI*) or vehicle (*V*) for 3, 6, 9, and 12 h as indicated, and the impact on the kinase upstream from MAPK, MEK, was assayed using phosphospecific MEK antibodies. *M*, mock-treated. Loading was confirmed by stripping and reprobing with an MEK antibody that was not phosphorylation state-dependent, and also by stripping and reprobing with an antibody to GRP-94. All panels in this figure are representative results from one of two independent experiments. *B*, A1N4-*myc* or MDA-MB-231 cells were preincubated with either okadaic acid (*OA*) at 30 nM, sodium orthovanadate (*OV*) at 1 mM for 60 min, or the two together (*OA*+*OV*), and then treated either with vehicle (−) or with Z-LLF-CHO at 10 μM (+) for 6 (A1N4-*myc*) or 12 (MDA-MB-231) h. Levels of dually phosphorylated ERK-1/2 were then determined by Western blotting. The -fold decrease of phospho-MAPK levels is shown in relation to vehicle-treated cells for Z-LLF-CHO, and for phosphatase-treated cells when combinations of phosphatase and proteasome inhibitors were used. Data were adjusted for loading of HSC-70. *C*, A1N4-*myc* or MDA-MB-231 cells were treated with controls or the proteasome inhibitors Z-LLF-CHO or lactacystin, as described in the legend to Fig. 1, and the impact on MKP-1 expression was assayed by Western blotting. Equal loading was confirmed by reprobing with antibodies that recognize HSC-70. The migration of pre-stained and biotinylated protein molecular size markers is indicated.

tions that would inactivate both PP2A and PP1 (49–51), sodium orthovanadate to inactivate tyrosine phosphatases (52, 53), or both, and then exposed to a proteasome inhibitor. Although okadaic acid itself caused a mild increase in phospho-ERK-1/2 levels, this inhibitor was unable to block proteasome inhibitor-induced dephosphorylation of p44/p42 MAPK in either A1N4-*myc* (Fig. 2*B*, *left panel*) or MDA-MB-231 cells (Fig. 2*B*, *right panel*). When calyculin A was used, another cell-permeable inhibitor of PP2A and PP1 (54), it also proved unable to block inactivation of ERK-1/2 (results not shown), further supporting that this dephosphorylation did not occur predominantly through a PP1- or PP2A-dependent mechanism. Sodium orthovanadate, in contrast, not only increased the baseline levels of phospho-ERKs, but also significantly inhibited the ability of proteasome inhibition to induce ERK dephosphorylation in A1N4-*myc* cells (Fig. 2*B*, *left panel*), and almost completely did so in MDA-MB-231 cells (Fig. 2*B*, *right panel*). These results suggested that induction of a vanadate-sensitive, but okadaic acid-insensitive, protein-tyrosine phosphatase was responsible for MAPK dephosphorylation.

Although there are several dual specificity phosphatases involved in regulation of MAPK activity (47, 48), mitogen-activated protein kinase phosphatase-1 (MKP-1) seemed worthy of special attention because it is induced by heat shock and genotoxic stress (55–57), and proteasome inhibition activates the heat shock response (22, 58). In addition, the nonspecific inhibitor ALLN has been reported to increase MKP-1 expression and ubiquitin conjugates of MKP-1, supporting a role for the proteasome in MKP-1 degradation (36), although in another study ALLN was unable to increase MKP-1 levels (35). The expression of MKP-1 in A1N4-*myc* and MDA-MB-231 cells exposed to Z-LLF-CHO was therefore evaluated by Western blotting. Compared with mock-, vehicle-, and control peptide-treated cells, all of which had low levels of MKP-1 expression, in both A1N4-*myc* (Fig. 2*C*, *left panel*) and MDA-MB-231 cells (Fig. 2*C*, *right panel*) a significant induction of MKP-1 was noted. To determine whether proteasome inhibition alone was sufficient to induce MKP-1, lactacystin was used and it was also able to increase cellular levels of this phosphatase. Moreover, there appeared to be a correlation between MKP-1 expression and phospho-MAPK content, because MDA-MB-231 cells treated with lactacystin had the lowest MKP-1 expression and had the least dephosphorylation of ERK-1/2 (compare Figs. 2*C* (*right panel*) and 1*A* (*right panel*)).

MKP Induction Correlates with MAPK Dephosphorylation—To further probe the possible link between proteasome inhibitor-induced MKP-1 expression and dephosphorylation of p44/42 MAPK, the concentration and time dependence of MKP-1 induction was studied. In both A1N4-*myc* (Fig. 3*A*, *left panel*) and MDA-MB-231 cells (Fig. 3*A*, *right panel*), MKP-1 was induced in a manner dependent on the concentration of Z-LLF-CHO. Inhibition of the proteasome alone was sufficient for this induction to be seen, because lactacystin demonstrated a similar ability (Fig. 3*B*, *left* and *right panels*, respectively). During time-course experiments, Z-LLF-CHO was noted to induce MKP-1 expression as early as after 3 h, with a subsequent increase in a time-dependent manner in A1N4-*myc* (Fig. 3*C*, *left panel*) and MDA-MB-231 cells (Fig. 3*C*, *right panel*). Lactacystin induced MKP-1 by 3 h as well, but unlike for Z-LLF-CHO, this induction then peaked at 6 h, and slowly declined subsequently (Fig. 3*D*, *right* and *left panels*). A comparison of MKP-1 induction (Fig. 3) with p44/42 MAPK dephosphorylation (Fig. 1) showed that both increased in a proteasome inhibitor concentration-dependent fashion, although a more continuous induction of MKP-1 was seen. Phospho-ERK-1/2 levels appeared to reach a nadir value, and some dually phosphorylated kinase was always detectable. Comparing the time-course studies, MKP-1 induction appeared to somewhat precede p44/42 MAPK dephosphorylation and, in the case of lactacystin, when MKP-1 levels declined beginning at 9 and then 12 h, phospho-MAPK levels recovered. These findings are consistent with the hypothesis that induction of MKP-1 is responsible in part for the dephosphorylation of ERK-1 and -2.

To more directly probe the relationship between MKP induction and MAPK dephosphorylation, antisense oligonucleotides and cell lines expressing MKP-1 in an antisense direction were employed. Both of these strategies resulted in only modest inhibition of MKP-1 expression, which mildly decreased MAPK dephosphorylation (results not shown). Because induction of MKP-1 in other systems has been noted to be dependent on gene expression, it therefore can be quantitatively inhibited by actinomycin D (59) and Ro-31-8220 (60). The effect of these agents on proteasome inhibitor-induced MKP-1 expression was therefore evaluated by preincubating cells prior to addition of Z-LLF-CHO. Actinomycin D completely blocked the ability of the proteasome inhibitor to induce MKP-1 in both A1N4-*myc* (Fig. 3*E*, *left panel*) and MDA-MB-231 (Fig. 3*E*, *right panel*) cells, as did Ro-31-8220. Both compounds increased the basal levels of dually phosphorylated ERK-1 and -2 compared with vehicle-treated controls. Furthermore, in the course of preventing induction of MKP-1, these agents significantly blocked the ability of the proteasome inhibitor to induce dephosphorylation of p44/42 MAPK, suggesting that MKP-1 was a major contributor to this inhibitor-mediated activity. When lactacystin was

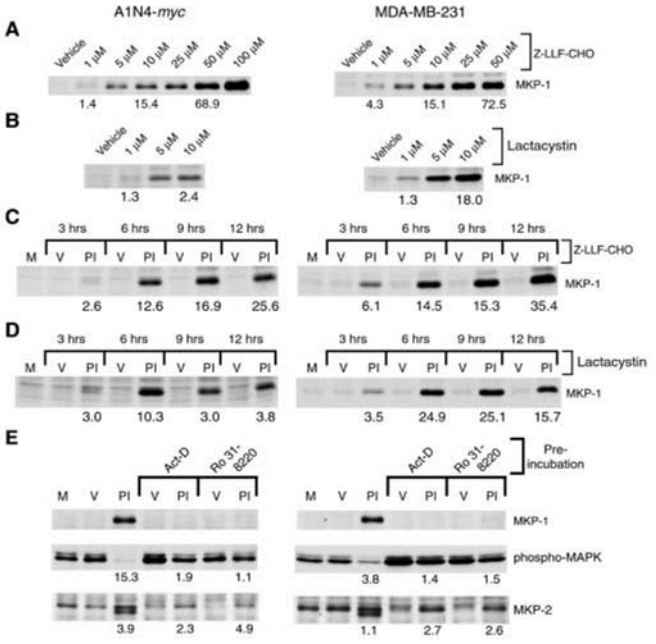

FIG. 3. **MKP expression in cells treated with proteasome inhibitors.** *A*, A1N4-*myc* or MDA-MB-231 cells were exposed to the indicated concentrations of Z-LLF-CHO for either 6 (A1N4-*myc*) or 12 (MDA-MB-231) h. The impact on MKP-1 expression was assayed by Western blotting, and loading was confirmed by stripping and reprobing with HSC-70 antibodies (data not shown). The -fold induction of MKP-1 is shown in relation to vehicle-treated cells and is adjusted for HSC-70. All *panels* are representative results from one of two independent experiments. *B*, A1N4-*myc* or MDA-MB-231 cells were exposed to the indicated concentrations of lactacystin for either 9 (A1N4-*myc*) or 12 (MDA-MB-231) h. The -fold induction of MKP-1 is shown. *C* and *D*, A1N4-*myc* or MDA-MB-231 cells were exposed to 5 μM Z-LLF-CHO (*panel C*) or 5 μM lactacystin (*panel D*) for the indicated times, whereas mock-treated cells (*M*) were fed with fresh medium for 5 min. The -fold induction of MKP-1 is shown in relation to vehicle-treated cells harvested in parallel at each time point and is adjusted for loading of HSC-70. *E*, A1N4-*myc* or MDA-MB-231 cells were preincubated for 30 min with 3 μg/ml actinomycin D or 5 μM Ro-31-8220. They were then treated with Z-LLF-CHO (*PI*) at 10 μM or vehicle (*V*) for 6 h, in parallel with mock-, vehicle-, and inhibitor-treated cells without actinomycin D or Ro-31-8220. Each *upper panel* shows the impact on MKP-1 expression, as determined by Western blotting. The *middle panels* show phospho-ERK-1/2 content, with the -fold decrease of phospho-MAPK levels indicated in relation to vehicle-treated cells for Z-LLF-CHO, and for actinomycin D (*Act-D*)- or Ro-treated cells when combinations were used, all with corrections for loading of HSC-70. MKP-2 expression is shown in the *lower panels*, which were generated by stripping and reprobing the MKP-1 blots with anti-MKP-2 antibody. This latter reagent has some cross-reactivity with MKP-1, which can be seen in those cells treated with the proteasome inhibitor alone. Induction of MKP-2 is shown in relation to HSC-70 as a loading control.

used in these assays, actinomycin D and Ro-31-8220 again blocked MKP-1 expression and inhibited MAPK dephosphorylation (results not shown).

Although proteasome inhibitor-mediated MAPK inactivation could be significantly decreased by blocking MKP-1, some decrease in phospho-MAPK levels was still notable, suggesting the presence of another phosphatase. Expression of the related dual specificity phosphatase MKP-2 under these conditions was therefore evaluated because it has been reported to be induced by ALLN (36). MKP-2 was noted to be present in both A1N4-*myc* (Fig. 3*E*, *left panel*) and MDA-MB-231 (Fig. 3*E*, *right panel*) cells that were mock- and vehicle-treated. Proteasome inhibition with Z-LLF-CHO resulted in a mild induction of MKP-2 in both cell lines, which was much more modest than that seen with MKP-1. Actinomycin D and Ro-31-8220 both decreased MKP-2 levels, but subsequent addition of a proteasome inhibitor was still able to increase the expression of this phosphatase. These results support the hypothesis that MKP-1 is the major agent of MAPK dephosphorylation induced by proteasome inhibition, but other phosphatases such as MKP-2 may play a role as well.

MAPK Blockade Enhances Proteasome Inhibitor-mediated Apoptosis—The p44/42 MAPK pathway plays a role in apoptosis (28–30), and it was therefore of interest to examine the possibility that proteasome inhibitor-mediated loss of phospho-ERK-1/2 was involved in the induction of programmed cell death. Because MEK activity had not been affected by proteasome inhibitors (Fig. 2*A*), the possibility was considered that MEK inhibition, resulting in a further decrease in phospho-MAPK levels, would increase apoptosis. A1N4-*myc* (Fig. 4*A*) and MDA-MB-231 cells (Fig. 4*B*) were therefore preincubated with one of two structurally unrelated MEK inhibitors, either PD 98059 (61, 62) or U0126 (63), and then exposed to Z-LLF-CHO or lactacystin. The MEK inhibitors themselves had a small impact on apoptosis compared with vehicle controls. PD 98059, for example, increased apoptosis of A1N4-*myc* cells by only 1.06-fold, whereas apoptosis of MDA-MB-231 cells was increased by 1.11-fold (results not shown). Both proteasome inhibitors had a much greater impact on apoptosis, with lactacystin increasing apoptosis in A1N4-*myc* cells by 1.38-fold, and in MDA-MB-231 cells by 2.34-fold (results not shown). When the combination of these agents with proteasome inhibitors was compared with proteasome inhibitors alone, however, a significant increase in apoptosis was noted in both A1N4-*myc* (Fig. 4*A*) and MDA-MB-231 cells (Fig. 4*B*). Combinations of lactacystin and PD 98059 enhanced apoptosis by 1.87-fold in A1N4-*myc* cells, and 3.02-fold in MDA-MB-231 cells. U0126 was even more active in this regard, in that with lactacystin it increased apoptosis by 2.65- and 3.89-fold in these two cell lines, respectively (Fig. 4, *A* and *B*). Analysis of phospho-MAPK levels revealed that the combination of an MEK inhibitor and a proteasome inhibitor resulted in further decreases in the abundance of dually phosphorylated ERK-1/2 than was induced by the proteasome inhibitor alone (results not shown).

PD 98059 and U0126 are fairly specific inhibitors of MEK, but the possibility was considered that their enhancement of proteasome inhibitor-mediated apoptosis could be occurring through effects other than those on the p44/42 MAPK pathway. To evaluate this, cell lines were prepared that expressed the K52R ERK-2 mutant, the kinase activity of which is ~5% of that of wild-type ERK-2 (44). Both the vector-transfected (A1N4-*myc*/pLPCX) and dominant negative (A1N4-*myc*/pLPCX-ERK-K→R) cells underwent apoptosis after treatment with Z-LLF-CHO or lactacystin. Compared with A1N4-*myc*/pLPCX cells, however, the A1N4-*myc*/pLPCX-ERK-K→R cells underwent 2.31-fold more apoptosis after exposure to Z-LLF-CHO (Fig. 4*C*), and 1.38-fold more apoptosis after exposure to lactacystin (Fig. 4*C*). MDA-MB-231 clones expressing the K52R ERK-2 mutant could not be isolated, suggesting a greater dependence for survival on p44/42 MAPK function in these cells, but such lines were successfully prepared from BT-474 human mammary carcinoma cells (41). When these cells are exposed to proteasome inhibitors, levels of dually phosphorylated ERK-1 and -2 decline, whereas MKPs are induced, in a manner analogous to that described above for A1N4-*myc* and MDA-MB-231 cells (results not shown). BT-474 vector-transfected cells (BT-474/pLPCX) and those expressing dominant negative ERK-2 (BT-474/pLPCX-ERK-K→R) were induced into apoptosis by Z-LLF-CHO or lactacystin. The K52R ERK-2 mutants, however, underwent 2.07- and 1.71-fold more apoptosis after Z-LLF-CHO or lactacystin treatment, respectively, compared with the vector-transfected controls (Fig. 4*D*).

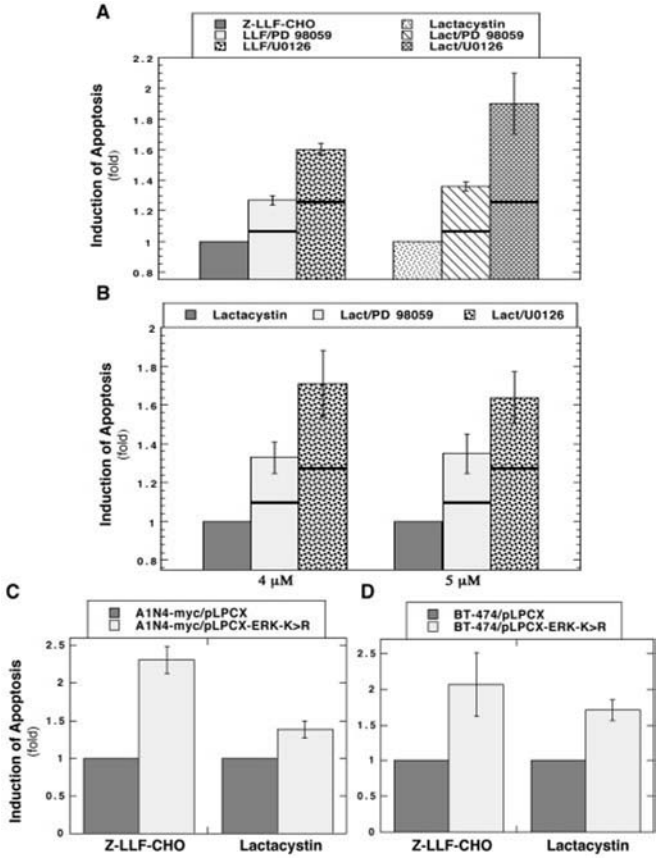

FIG. 4. **Impact of MAPK blockade on proteasome inhibitor-mediated apoptosis.** *A*, A1N4-*myc* cells were preincubated with either 20 μM PD 98059 or U0126 for 2 h and then treated with 3 μM Z-LLF-CHO or 4 μM lactacystin for an additional 6 h. The extent of any induced apoptosis was determined using the Cell Death Detection ELISA^{PLUS} kit using vehicle-treated cells as controls. Apoptosis induced by either of the two proteasome inhibitors alone in A1N4-*myc* cells was then arbitrarily set at 1.00, and apoptosis induced by combinations with PD 98059 or U0126 is expressed in relation to that value as a -fold increase over 1.00. Note that the results described in the text are expressed as -fold induction compared with vehicle controls. A *black bar* represents the expected impact of PD 98059 or U0126 alone based on control experiments, and any excess apoptosis above that level suggests a synergistic interaction between the proteasome and MEK inhibitors. All *panels* represent the results of four experiments, and the mean -fold increase in apoptosis is shown along with the standard error of the mean. *B*, MDA-MB-231 cells were preincubated with either 20 μM PD 98059 or U0126 for 2 h and then treated with 4 or 5 μM lactacystin for 12 h. The -fold induction of apoptosis over controls was evaluated, calculated, and expressed as above. *C*, A1N4-*myc* cells containing either the pLPCX vector, or those expressing the dominant negative K52R ERK-2 mutant, were treated with Z-LLF-CHO or lactacystin, and the induction of apoptosis was compared with vehicle-treated cells. The results in pLPCX-containing cells were then arbitrarily set at 1.00, and programmed cell death induced in mutant ERK-containing cells was expressed as the -fold increase compared with 1.00. To rule out the possibility of clonal variation, two separate single-cell clones were assayed and the results presented are the mean of the two. *D*, pooled clones of BT-474 cells containing either pLPCX or the K52R ERK-2 mutant were treated with 10 μM Z-LLF-CHO or 20 μM lactacystin for 18 h. The results were analyzed and are presented as described in *panel C*.

MAPK Activation Decreases Proteasome Inhibitor-mediated Apoptosis—If proteasome inhibitor-mediated apoptosis occurs in part through decreased p44/42 MAPK signaling, then increased activation of this pathway should decrease apoptosis. To evaluate this possibility, A1N4-*myc* and MDA-MB-231 cells were exposed to Z-LLF-CHO or lactacystin either in the presence or absence of EGF, which stimulates the ERK-1/2 signal transduction cascade (reviewed in Refs. 64 and 65). In A1N4-*myc* cells epidermal growth factor decreased Z-LLF-CHO-mediated apoptosis by 2.44-fold and lactacystin-induced apoptosis by 1.39-fold (Fig. 5*A*). In MDA-MB-231 cells, this effect of EGF was 1.69-fold for both proteasome inhibitors. EGF can also activate other anti-apoptotic pathways in some breast cancer cells, such as the protein kinase B/Akt serine/threonine kinase (66). Cell lines expressing the D319N ERK-2 sevenmaker mutant that has significantly reduced sensitivity to dephosphorylation by MKP-1 and -2 (43) were therefore prepared. Expression of this dominant-positive allele in A1N4-*myc* cells inhibited apoptosis by 1.43- and 1.30-fold when they were treated with Z-LLF-CHO and lactacystin, respectively, compared with vector-containing controls (Fig. 5*C*), whereas in MDA-MB-231 cells lactacystin-induced apoptosis was inhibited by 1.52-fold (Fig. 5*D*). The finding that blockade of MAPK activity using either pharmacologic or dominant-negative constructs enhances apoptosis, whereas MAPK activation with EGF or dominant positive mutants inhibits programmed cell death, strongly supports the hypothesis that inhibition of signaling through p44/42 MAPK plays a role in proteasome inhibitor-mediated apoptosis.

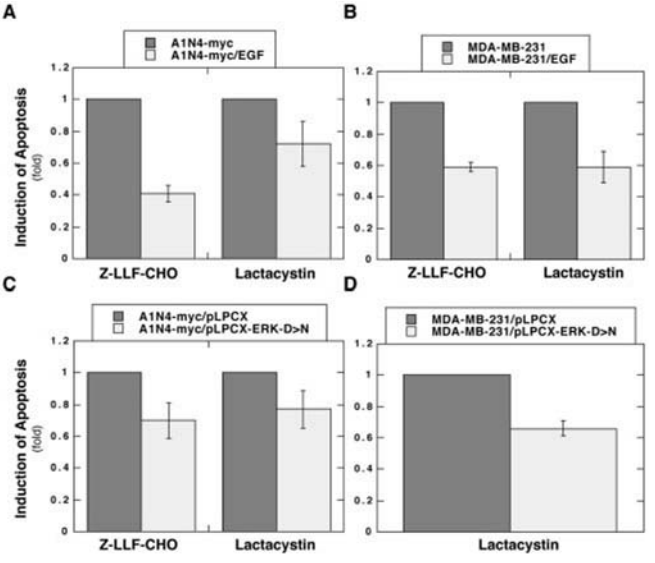

FIG. 5. **Impact of increased MAPK activity on proteasome inhibitor-mediated apoptosis.** *A* and *B*, A1N4-*myc* (*panel A*) or MDA-MB-231 cells (*panel B*) were exposed to Z-LLF-CHO or lactacystin, as described in the legend to Fig. 4, either in complete medium or medium supplemented with EGF at 10 ng/ml. Apoptosis induced in the presence of complete medium was arbitrarily set at 1.00, and programmed cell death in the presence of EGF was expressed in relation to this value. The -fold inhibition referred to in the text is the inverse of the values presented in the *graph*. All *panels* represent the results of three to four experiments performed in duplicate. *C* and *D*, The impact of the phosphatase-resistant ERK sevenmaker mutant on proteasome inhibitor-mediated apoptosis was evaluated in A1N4-*myc* (*panel C*) and MDA-MB-231 cells (*panel D*), with apoptosis of vector-containing cells arbitrarily set at 1.00 as described in Fig. 4. Two separate single cell clones were evaluated, and the results were combined. Z-LLF-CHO was not tested in the MDA clones.

DISCUSSION

The ability of proteasome inhibitors to induce apoptosis in tumor-derived cells lines and animal models of human malignancies (10–12) has sparked interest in their use as agents in cancer therapeutics (reviewed in Ref. 67). A better understanding of the underlying mechanisms by which proteasome inhibitors act will allow a rational use of these compounds and contribute to the design of novel combination regimens. Proteasome inhibitors have been reported to inhibit nuclear translocation and signaling through the NF-κB pathway (10, 11),

and to activate signaling through the c-Jun N-terminal kinase and p38 MAPKs (22). Their effects on another ubiquitous signaling cascade, the p44/42 MAPK or ERK-1/2 pathway, have not been well characterized, however, with proteasome-specific inhibitors. Although activity of this pathway is associated with cellular proliferation and apoptosis in many cells (28–30), its importance has been particularly well studied in breast cancer. Signaling by the *erb*B receptor tyrosine kinase family, which occurs in part through p44/42 MAPK, has been implicated in the development and progression of breast cancer, and high expression of *c-erb*B-2 (HER-2/neu) and the homologous EGF receptor (*c-erb*B-1) is a poor prognostic sign (reviewed in Ref. 68). Indeed, elevated activity of ERK-1/2 alone has been suggested to have prognostic significance for disease-free survival of breast cancer patients (69). Given this critical role for MAPK activity in breast cancer, breast epithelium- and tumor-derived cell lines were chosen as the subjects of this study.

Inhibition of the proteasome with two structurally distinct inhibitors, including the tripeptidyl aldehyde Z-LLF-CHO and the *Streptomyces* product lactacystin, resulted in decreased levels of dually phosphorylated ERK-1 and -2 (Fig. 1*A*) in a concentration- and time-dependent fashion (Fig. 1, *B–E*). This decrease took place despite the continued presence of either fetal bovine serum in the case of MDA-MB-231 cells, or even serum supplemented with EGF in the case of A1N4-*myc* cells. A recent investigation of multiple myeloma cells treated with one concentration of PS-341 found that activation of phospho-MAPK by interleukin 6 or binding to bone marrow stroma was blocked by this proteasome inhibitor (37). Because this compound (16), like Z-LLF-CHO (38, 45) and lactacystin (46), is able to inhibit the chymotrypsin-like activity of the proteasome, it seems that blockade of ERK-1/2 signaling may be a common property of proteasome inhibitors. One possible exception was recently reported by Vrana and Grant (70), who noted that lactacystin did not induce ERK phosphorylation in U937 monocytic leukemia cells. However, at the concentration of 1.0 μM used in their study, we also found a small impact on MAPK (Fig. 1*C*), and it is therefore possible that higher lactacystin concentrations would have a similar effect on U937 cells. A second possible exception is the work of Torres *et al*. (35), who demonstrated that the nonspecific inhibitor ALLN at 100 μM did not decrease dually phosphorylated MAPK levels in Balb/c 3T3 cells. They evaluated cells after a 6-h exposure, however, and in our studies we also found that Z-LLF-CHO had only slight effects on MAPK at 6 h in MDA-MB-231 cells (Fig. 1*D*, *right panel*), suggesting that a longer exposure in their system could have uncovered a loss of active MAPK.

Although phospho-p44/42 MAPK levels were decreased by proteasome inhibition the activity of the MEK kinase appeared not to be affected (Fig. 2*A*), suggesting that MAPK dephosphorylation was because of a phosphatase. Several lines of evidence implicated MKPs as the major phosphatases involved in this process. MKP-1 is induced by heat shock and genotoxic stress (55–57), whereas proteasome inhibitors activate the heat shock response (22, 58). In addition, the nonspecific proteasome inhibitor ALLN was reported to increase MKP-1 and -2 expression, along with ubiquitin-MKP-1 conjugates (36), supporting a role for the proteasome in MKP turnover. We found that MKP-1 was strongly induced by both Z-LLF-CHO and lactacystin (Fig. 2*C*), demonstrating that inhibition of the proteasome alone was sufficient to increase MKP-1 expression. This occurred in a concentration- and time-dependent manner (Fig. 3, *A–D*) that was consistent with an effect on phospho-MAPK levels. Quantitative blockade of MKP-1 induction using either actinomycin D or Ro-31-8220 almost completely preserved the activity of ERK-1/2 (Fig. 3*E*). The experiments with actinomycin D demonstrate for the first time that, whereas some of the proteasome inhibitor-mediated increase in MKP-1 expression may be because of protein stabilization, a sizable proportion of it is likely because of induction of MKP transcription. Because MKP-1 is widely expressed (47, 48), it may also be responsible for the ability of PS-341 to inhibit interleukin 6-induced MAPK activation in myeloma cells (37), although mechanistic studies were not reported by the authors. A possible exception is provided by the work of Torres *et al.* (35), who found no induction of MKP-1 in their Balb/c 3T3 system after 6 h. It is again possible that longer exposures might uncover MKP induction, or that this may prove to be a cell type-specific effect. In addition, the possibility that other proteasome inhibitor-induced phosphatases play a role in MAPK inactivation cannot be excluded. Expression of the MKP-3 and VHR dual-specificity phosphatases was not increased in these cells (results not shown), but the serine/threonine protein phosphatase 5 is reportedly induced by proteasome inhibition in MCF-7 breast carcinoma cells (71). A combination of these and other phosphatase activities may be required to fully dephosphorylate ERK-1 and -2.

Given the central role of the proteasome in normal cellular homeostasis, the pro-apoptotic effects of proteasome inhibitors are likely to be because of an impact on several important pathways. Because signaling through the Ras/Raf/MEK/ERK cascade is crucial to many cellular processes, however, inhibition of p44/42 MAPK activity may prove to be one of the more important effects of these inhibitors. To evaluate this possibility, we first postulated that, if MAPK inhibition was important to proteasome inhibitor-mediated apoptosis, further MAPK blockade should enhance programmed cell death. This proved to be the case, because the MEK inhibitors PD 98059 and U0126 enhanced both Z-LLF-CHO- and lactacystin-induced apoptosis in A1N4-*myc* (Fig. 4*A*) and MDA-MB-231 cells (Fig. 4*B*), and a dominant negative ERK-2 construct showed a similar impact (Fig. 4, *C* and *D*). We then postulated that, if MAPK inhibition played a role in proteasome inhibitor-mediated apoptosis, opposing this inactivation should inhibit programmed cell death. MAPK stimulation with EGF did indeed inhibit the induction of programmed cell death (Fig. 5, *A* and *B*), as did the use of a mutant MAPK that was resistant to dephosphorylation by MKPs (Fig. 5, *C* and *D*). Dysregulation of MAPK function therefore appears to be an important step for proteasome inhibitor-mediated apoptosis in these cell lines. Recent work by Vrana and Grant (70) also concluded that the MAPK cascade was an important target during the synergistic induction of apoptosis in U937 cells treated with a combination of bryostatin and lactacystin. They found, however, that lactacystin prolonged the activation of MAPK induced by the protein kinase C activator bryostatin when the two agents were used concurrently, and that MEK inhibitors attenuated apoptosis induced by this regimen (70). Because the impact of MEK inhibitors and lactacystin was not studied in the absence of bryostatin, it is possible that this difference is because of a specific interaction of proteasome inhibitors with the latter agent. Alternatively, it may point to a cell type-specific effect, which, if documented, would have important clinical implications, because it would suggest that a proteasome inhibitor/MEK inhibitor regimen would enhance tumor killing in patients with breast cancer, but might antagonize it in those with monocytic leukemia.

In addition to shedding light on the potential mechanisms by which proteasome inhibitors affect tumor growth, the current study allows the formation of hypotheses about their optimal use in a clinical setting. Breast tumors overexpressing *c-erb*B-2 and *c-erb*B-1 might have been hypothesized to be more susceptible to proteasome inhibitor-mediated apoptosis because of a

greater reliance on the survival signals communicated through the Ras/Raf/MEK/MAPK pathway, and the ability of proteasome inhibitors to decrease MAPK activity. The studies with EGF, however, suggest the opposite, in that MAPK stimulation opposed the induction of programmed cell death. If this proves to be the case in *in vivo* models, these inhibitors should be targeted especially toward patients with low expression of *c-erb*B-2 and *c-erb*B-1, whereas those with high expression might benefit from a proteasome inhibitor in combination with an MEK inhibitor. Such a regimen would represent a welcome addition to the chemotherapeutic armamentarium, because these patients have a poorer prognosis with currently available agents (68). The ability of proteasome inhibitors to block MAPK activity also suggests that they should be effective in combination with Taxol®, another agent used in patients with breast cancer. This inhibitor of microtubule assembly has been recently reported to activate MAPK, and pharmacologic agents such as U0126 that inhibited MEK activity and MAPK phosphorylation enhanced Taxol®-induced cytotoxicity (72). Although proteasome inhibitors inactivate MAPK through a different mechanism, they should similarly enhance the antitumor efficacy of Taxol®, suggesting that trials of this combination in patients with breast cancer are warranted.

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