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p53-dependent apoptosis induced by proteasome inhibition in mammary epithelial cells

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Abstract

We have examined the effects of inhibition of the 26S proteasome in a murine mammary cell line, KIM-2 cells using the peptide aldehyde inhibitor MG132. These studies have demonstrated a clear requirement for proteasome function in cell viability. Induction of apoptosis was observed following MG132 treatment in KIM-2 cells and this death was shown to be dependent on the cell actively traversing the cell cycle. KIM-2 cells were generated using a temperature sensitive T-antigen (Tag) and studies at the permissive temperature (33°C) have shown that a Tag binding protein was essential for this apoptotic response. Studies in two additional cell lines, HC11, which is a mammary epithelial cell line carrying mutant p53 alleles and p53 null ES cells suggest that p53 is actively required for the apoptosis induced as a consequence of proteasome inhibition. These results suggest a pivotal role for the 26S proteasome degradation pathway in progression through the cell cycle in proliferating cells. *Cell Death and Differentiation* (2001) **8,** 210–218.

Keywords: KIM-2 mammary epithelial cells; p53; apoptosis; 26S proteasome

Abbreviations: ALLM, Ac-Leucinyl-leucinyl-methional; E64, trans-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester; GF, growth factor; MG132, Cb_2-leucinyl-leucinyl-leucinal; PMSF, phenylmethylsulphonyl fluoride

Introduction

The normal growth and metabolism of cells are dependent not only on the presence and activation of critical proteins at specific times but also on their timely removal, thus allowing an ordered chain of events, i.e. cell cycle progression. A key player in the degradation of proteins within the cell is the 26S proteasome. The vast majority of intracellular degradation is mediated by the 26S proteasome and it is involved in selective turnover of proteins under basal metabolic conditions. Cells are often required to switch from one cellular state to another in response to environmental signals or as part of regulated developmental pathways. Switches of this manner require the rapid dismantling of existing regulatory networks, which are often dependent on protein degradation. The process of selective degradation has two major advantages: it is fast, and it is irreversible, thereby ensuring no inappropriate reactivation. These features explain why selective degradation plays such a prime regulatory role in systems which rely on timing controls.

The 26S proteasome complex is composed of a central 20S core which contains all the proteolytic activity of the complex, and a regulatory 19S cap complex. The core complex is composed of seven different α and β subunits, which form four rings each containing all seven subunits. The 19S cap complex is composed of 15 different subunits ranging in size from 25 to 110 kDa. In association with the 20S proteasome, the 19S cap complex confers an ATP dependence on protein degradation. Within the cap there are six Mg^{2+}-dependent ATPases which are thought to function in the unfolding and transportation of the substrate protein towards the proteolytic sites within the inner channel of the 20S core.[1] Proteins which are targeted for degradation are identified by attachment of a polyubiquitin tail, a process itself catalysed by a complex cascade of enzymes. Of the non-ATPase subunits in the cap complex, one protein (S5) has been shown to bind multi-ubiquitin chains and therefore appears to be responsible for recognition of the ubiquitinated substrate protein by this complex.[2] A number of natural substrates of this pathway exist. These range from proteins as diverse as the transcription factor NFκB to the cyclins. Degradation of the NFκB inhibitor, IκBα allows activation of this transcription factor by relieving cytoplasmic tethering, whereas degradation of the B type cyclins permits cells to exit mitosis and divide.[3,4]

By its very nature the 26S proteasome could be expected to play a pivotal role in the control of normal cell processes of proliferation, differentiation and apoptosis. Apoptosis is the process whereby a cell undergoes a genetic program resulting in self destruction.[5] It is a widespread phenomenon that underlies both normal development and pathogenesis. The molecular events controlling this process at present remain unclear, but more recently the proteolytic events occurring during the apoptotic cascade have become the prime focus of this research field. It has become apparent that the activation of

proteolytic enzymes is paramount to the apoptotic process.[6] The major proteases involved in apoptosis are a family of cysteine proteases called Caspases. These proteins are activated by proteolytic cleavage of proenzymes and thus trigger a multi-enzyme cascade that drives apoptotic events. Any role that the proteasome may play during an apoptotic cascade is currently unclear.

The mammary gland is a unique model system to study as it has the ability to undergo successive rounds of proliferation, differentiation and apoptosis. It is therefore one of the few tissues in which questions relating to all three of these processes can be addressed. In this study the model of mammary development used was KIM-2 cells.[7] Using a differential display approach we had previously observed upregulation of some proteasome components during proliferation of the gland and for these reasons we decided to examine the consequences of proteasome disruption within an actively dividing cell. Using the KIM-2 mammary epithelial cell line we have demonstrated an absolute requirement for a functional 26S-degradation pathway during proliferation. Inhibition of this pathway was observed to induce apoptosis that was dependent on the presence of an SV40 Tag binding protein. Studies with p53null ES cells demonstrated that p53 was essential for cell death induced by proteasome inhibition. This study thus extends our knowledge of the essential role the proteasome plays in maintaining a healthy cellular environment.

Results

Proteasome inhibition induces apoptosis

The KIM-2 mammary epithelial cell line was used in this study. As anticipated molecular markers of mammary differentiation were identified in KIM-2 cells cultured under appropriate conditions.[7] Thus these cells represent a good *in vitro* model in which to study mammary epithelial development. The peptide aldehyde inhibitor of the proteasome, MG132 selectively inhibits the 26S proteasome.[8] To investigate the role of the proteasome in apoptosis, we examined the effect of MG132 treatment on KIM-2 cells. Figure 1 shows results from KIM-2 cells treated with MG132, and harvested over 24 h. These results show that apoptotic cells can be observed within 4 h of MG132 treatment. The level of apoptosis increases until 18–20 h where it reaches a maximum of 20%. This is seen to correlate with a cell division time of 24 h in this cell line. We then examined the effects of a range of protease inhibitors on KIM-2 cells to determine if the apoptosis observed was specific to proteasome inhibition. The results shown in Table 1 were determined from morphological analysis of acridine orange stained cells. Apoptosis was identified by the presence of membrane blebbing, cytoplasmic shrinkage, and nuclear fragmentation. The cysteine/serine protease inhibitor, Leupeptin, the aspartic protease inhibitor, Pepstatin, and the serine protease inhibitor, PMSF did not induce apoptotic cell death at high concentrations over a 24 h period.[9–11] Similarly, the lysosomal cathepsin inhibitor, E64 did not induce apoptosis in KIM-2 mammary epithelial cells. In contrast to this, the calpain II inhibitor ALLM induced a low level of apoptosis (13%) at a high concentration (100 μM) over the course of the experiment. Two μM of MG132 induced $>25\%$ apoptosis over an identical time period. ALLM is well documented to cross-react with the proteasome as well as calpains.[8] E64 has cross-reactive inhibitory activity on calpains but did not induce apoptosis, therefore it seems highly likely that the death observed with high concentrations of ALLM treatment is due to its inhibitory activity on the 26S proteasome rather than the inhibition of calpains. In addition a second 26S proteasome inhibitor, Lactacystin was used and yielded very similar results to those observed with MG132, further evidence that the initiation of apoptosis was specific to the blockage of protein degradation by the 26S proteasome.

In KIM-2 cells we have shown that proteasome blockage induces an apoptotic response. In order to address whether proteasome inhibition could also play a protective role in

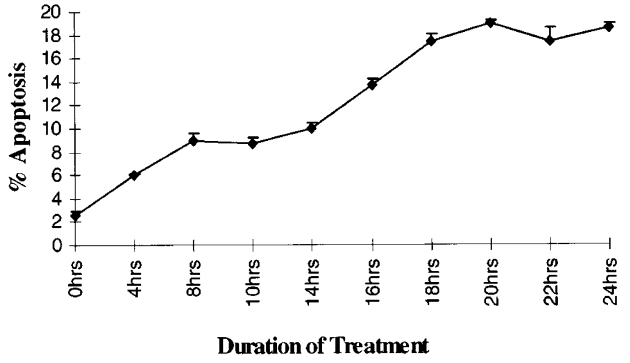

Figure 1 Annexin V time-course on KIM-2 cells. Cells were treated with MG132 over a 24 h period. Samples were harvested and an Annexin V assay performed. The results shown are the mean of three experiments $\pm$ S.E.M., and are expressed as the percentage of Annexin V positive cells

Table 1 Effect of a range of protease inhibitors on KIM-2 mammary epithelial cells

Treatment	% Apoptotic cells
Control	0.64
3% FCS	18.7
500 μM PMSF	0.8
500 μM Leupeptin	0.95
500 μM Pepstatin A	1.8
500 μM E64d	1.6
10 μM Calpain II inhibitor	2.3
25 μM Calpain II inhibitor	2.4
50 μM Calpain II inhibitor	5.7
100 μM Calpain II inhibitor	13.7
0.75 μM MG132	2.5
1 μM MG132	5.3
1.5 μM MG132	16.1
2 μM MG132	29.3
0.1 μM Lactacystin	2.8
1 μM Lactacystin	24.5
10 μM Lactacystin	37.8

The protease inhibitors PMSF, Leupeptin, Pepstatin A, E64d, Calpain II Inhibitor, MG132 and Lactacystin were added to media at various concentrations for 24 h. Cells were harvested and fixed for staining with acridine orange. The percentage death is shown as the mean of three independent experiments

mammary epithelial cells, KIM-2 cells were serum and growth factor (GF) starved to induce an apoptotic response. These cells were also treated with two concentrations of MG132 and the level of apoptosis analyzed. Annexin V staining determined that MG132 could not protect from apoptosis induced by GF withdrawal (Figure 2). The effects on death observed were seen to be additive. These experiments suggested that the proteasome was not involved in degrading and/or processing a protein that is essential for driving apoptosis induced by growth factor and serum starvation.

MG132 treatment induces apoptosis in a cell cycle dependent manner

To address the question of whether differences in cell cycle status might alter sensitivity to proteasome inhibition KIM-2 cells at different stages of confluency were treated with MG132 and death assessed morphologically by acridine orange staining. These results showed that in cells treated 4 h following passaging, MG132 addition for the following 24 h induced 50% apoptosis (Figure 3a). The level of death observed decreased as the cells became more confluent suggesting that the apoptosis induced had a dependency on the cells being within the cell cycle. KIM-2 cells reach confluency by 72 h which corresponds to the rate of apoptosis reaching a baseline level of approximately 20%. This was shown not to be the case for serum starved cells. A 10–15% death rate was observed with this death stimulus regardless of the confluency state of the cells.

To address whether KIM-2 cells were dying from a specific point in the cell cycle, we synchronised KIM-2 cells in G1 with the anti-metabolite Hydroxyurea and in G2/M with Nocodazole, a spindle poison. Cells were blocked for 24 h (equivalent to approximately one cell cycle) and then the drug was removed and replaced with either maintenance media (MM) or MM containing MG132. Cells were harvested and stained with annexin V for apoptosis analysis or fixed and stained with Propidium Iodide (PI) for cell cycle analysis. Results for cells blocked in G1/S phase showed that apoptosis was detectable within 2 h of MG132 treatment, and this increased dramatically by 4 h and thereafter increased slowly. In contrast to this, cells which had been blocked in G2/M showed a substantial increase in annexin V staining only at 12–16 h after treatment with MG132 (Figure 3b). Analysis of the cell cycle kinetics demonstrated that in cells released from G1 synchronisation, MG132 treatment caused an accumulation of cells in the G1/S-phase of the cell cycle (Table 2). KIM-2 cells synchronised in G2 prior to proteasome inhibition appeared to progress through G2/M but at a much slower rate than cells which were released from the block into maintenance media. The MG132 treated cells did not begin to accumulate in G1/S-phase until 10–12 h

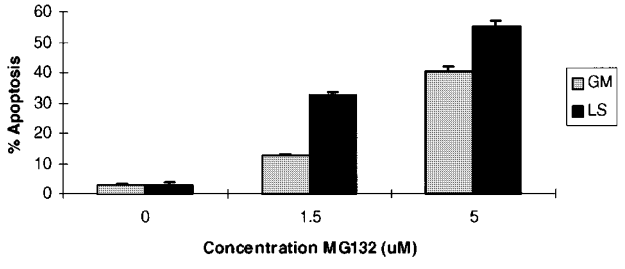

Figure 2 Can proteasome inhibition protect from apoptosis? Confluent KIM-2 cells were treated with either Growth media (GM), low serum media (LS) or with two different concentrations of MG132 (1.5 and 5 μM) for 24 h alone to induce apoptosis. Test samples were treated with 3% FCS and simultaneously with either concentration of MG132 to determine if proteasome inhibition could protect from apoptosis induced by growth factor and serum withdrawal. Samples were harvested at 24 h and an Annexin V assay was performed. Results shown are expressed as means ± S.E.M.

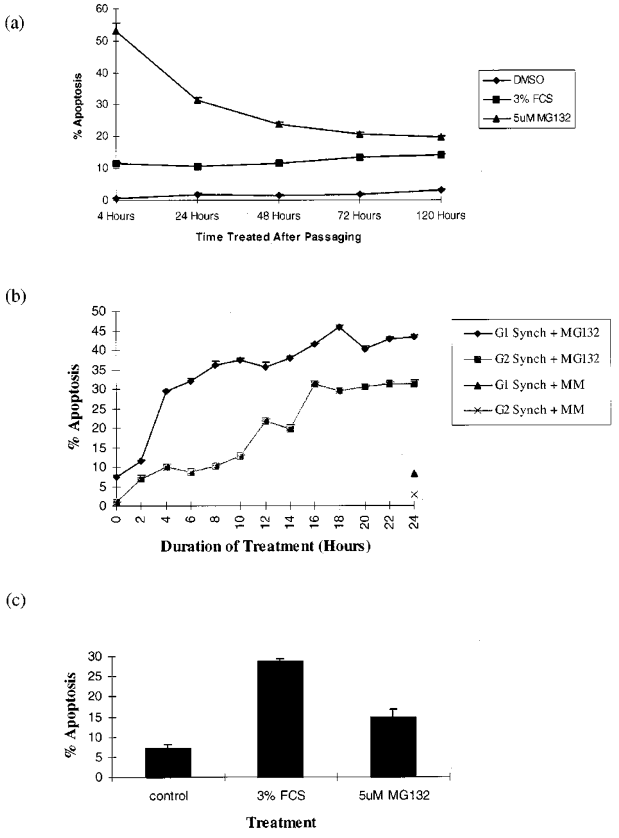

Figure 3 (**a**) Apoptosis analysis in a proliferating cell population. KIM-2 cells were passaged and treated with either DMSO, or serum and growth factor deprived, or cultured in the presence of 5 μM MG132, at 4, 24, 48, 72, and 120 h after passaging. Cells were treated for a further 24 h and an Annexin V assay performed. Results are shown as the percentage of Annexin V positive cells, as the mean of three experiments ± S.E.M. Cells were confluent at the 72 h stage. (**b**) Apoptosis induction as a consequence of proteasome inhibition following cell cycle synchronisation in KIM-2 cells. KIM-2 cells were passaged and allowed to grow for 24 h. Cells were subsequently blocked in G1/S-phase or at the G2/M boundary with Hydroxyurea (1 mM) or Nocodazole (50 ng/ml) respectively for 24 h (approximate cell division time). Following synchronisation cells were washed to remove the blocking drugs and then treated with either maintenance media (MM) or MM supplemented with 5 μM MG132 over a 24 h period. Cells were harvested and an Annexin V assay performed. Results shown are expressed as the percentage of Annexin V positive cells. The data is the mean of three experiments ± S.E.M. (**c**) Effect of proteasome inhibition on differentiated KIM-2 cells. KIM-2 cells differentiated for 12 days in the presence of prolactin and dexamethasone were treated with either 3% FCS or 5 μM MG132 for 24 h. Cells were harvested and stained with annexin V. Results are expressed as the mean of three experiments ± S.E.M.

Table 2 Induction of cell cycle arrest following proteasome inhibition in KIM-2 cells

	Hydroxyurea + MM				Hydroxyurea + MG132			
	Pre-G1	G1	S	G2/M	Pre-G1	G1	S	G2/M
Treatment		(±S.E.M.)				(±S.E.M.)		
0 Hours	13 (0.3)	67.9 (1.3)	14 (0.2)	8 (0.4)	6 (0.4)	70 (1.7)	10 (0.5)	11 (0.3)
12 Hours	14 (0.4)	35 (0.6)	36 (0.8)	10 (0.6)	29 (0.8)	34 (0.9)	22 (0.2)	9 (0.3)
24 Hours	21 (0.5)	51 (1.3)	18 (0.3)	10 (0.2)	41 (1.8)	24 (0.7)	26 (0.5)	2 (0.1)
	Nocodazole + MM				**Nocodazole + MG132**			
	Pre-G1	G1	S	G2/M	Pre-G1	G1	S	G2/M
Treatment		(±S.E.M.)				(±S.E.M.)		
0 Hours	8 (0.1)	15 (0.3)	12 (0.3)	65 (1.6)	2 (0.2)	6 (0.7)	7 (0.6)	74 (2.1)
12 Hours	8 (0.2)	51 (0.3)	15 (0.1)	20 (0.2)	11 (0.5)	18 (0.8)	12 (1.2)	45 (0.9)
24 Hours	15 (0.3)	48 (0.4)	14 (0.5)	14 (0.2)	28 (0.8)	24 (0.5)	13 (1.5)	27 (0.6)

KIM-2 cells were treated for 24 h with either 1 mM Hydroxyurea or 50 ng/ml Nocodazole 24 h following passaging. Cells were subsequently washed to remove the blocking drugs and then treated with either maintenance media alone (MM) or MM supplemented with 5 μM MG132 over a 24 h time period. Cells were harvested and stained with Propidium Iodide for cell cycle analysis. The flow data shown describes the 0, 12 and 24 h treatments

following the onset of treatment. This corresponds to the onset of apoptosis in these cells. Taking the cell cycle and apoptosis results together it would appear that the 26S proteasome is implicated in functioning during mitosis as MG132-treated KIM-2 cells were less efficient at traversing this stage of the cell cycle than control cells. These results also detailed an absolute requirement for proteasome function at the G1/-S phase of the cell cycle as the cells appeared to arrest and undergo apoptosis either during or following this stage in the cycle.

To determine if cells which had exited the cell cycle had a requirement for proteasome function in survival, annexin V staining was performed on KIM-2 cells which had been differentiated for 12 days with lactogenic hormones and were thus no longer actively dividing. Death was seen to occur at very low levels in these differentiated cells (Figure 3c). This result would suggest that cell proliferation is a major component of sensitivity to proteasome inhibition.

T-antigen expression abrogates proteasome inhibition induced apoptosis

KIM-2 cells were originally generated using SV40 T-antigen (Tag) as the immortalising agent. At 33°C, Tag complexes with p53, the retinoblastoma gene product (Rb), and another pRb family member p107 resulting in a cell population with no functional p53 or pRb.[12] To address whether any of these Tag binding proteins were involved in the apoptotic cascade induced by proteasome inhibition KIM-2 cells were cultured at the permissive temperature of 33°C and the response of these cells to MG132 compared to cells cultured at 37°C. KIM-2 cells were routinely cultured at 37°C, which represents a semi-permissive state and cells switched to 33°C were passaged for 14 days prior to treatment. At 33 and 37°C, MG132 treatment was continued for up to 96 h and the cells analyzed for apoptosis by annexin V expression (Figure 4a). These results showed that both groups of cells were induced to undergo apoptosis following serum and growth factor withdrawal but only the cells at 37°C induced an apoptotic response following MG132 treatment. The difference in response observed between the two groups of cells cannot be due to the change in temperature alone and are assumed to be due to the sequestration of a Tag binding protein. Examination of the results from serum and growth factor starvation show that the rate of apoptosis induced by this stimulus was similar in both groups of cells after 48 h arguing against a slower rate of cell death in cells cultured at the lower temperature. KIM-2 cells cultured at 33°C increased in number over the 96 h treatment period suggesting that these cells are actively dividing and the lack of apoptosis following MG132 treatment was not a function of exit from the cell cycle. These results suggest a role for a Tag binding protein in the apoptotic response induced by proteasome inhibition.

Overexpression of p53 is sufficient to induce apoptosis in response to a range of cell insults.[13] It was assumed that p53 levels may have been elevated in KIM-2 cells following proteasome inhibition as this protein is also documented to be a substrate of the 26S proteasome.[14] An elevation in p53 protein alone could represent the apoptotic signal following MG132 treatment. We therefore examined the level of p53 protein in cells treated with MG132. At 24 h the levels of p53 protein are similar in control cells to cells treated with MG132, suggesting that there is no long-term accumulation of p53 in KIM-2 cells (Figure 4b). This result suggests that KIM-2 mammary epithelial cells are not undergoing apoptosis as a consequence of the perturbation of p53 expression.

Recent evidence has suggested a role for the proteasome in regulating the p53-responsive genes MdM2 and Bax.[15] To determine if these proteins were stabilised in KIM-2 cells following proteasome inhibition we examined their levels following Lactacystin treatment. Levels of p53, MdM2, and Bax did not accumulate following inhibition of proteasome function (Figure 4c). We also analyzed MdM2 and Bax protein levels in HC11 cells, which carry a mutant p53 allele[16] to determine if stabilisation of these proteins could occur in the absence of p53. Bax and MdM2 levels in HC11 cells were at the limits of detection (data not shown) and again did not appear to accumulate following proteasome inhibition. These results suggest that the 26S proteasome, in this system, does not influence the turnover of two downstream targets of p53.

Wild-type p53 is required for apoptosis induced by proteasome inhibition

To test the hypothesis that p53 function was essential for apoptosis induced by proteasome inhibition we obtained ES cells which contained wild-type p53 (E14 cells), and which were null for p53 (R72D27 cells).[15] ES cells were treated with three different concentrations of MG132 for up to 48 h and assayed for cell death and cell cycle status. The levels of apoptosis were greatly enhanced in the p53 wt cells as

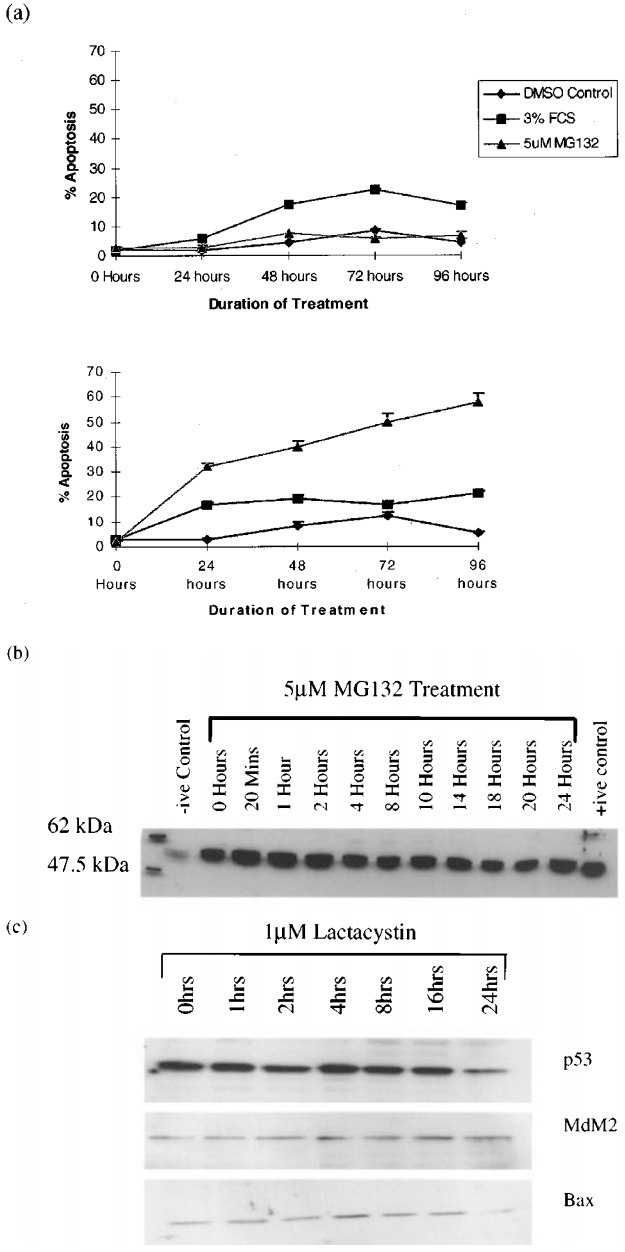

Figure 4 (**a**) A role for a Tag binding protein in apoptosis induced by proteasome inhibition. KIM-2 cells were cultured at either 33°C (upper panel) or 37°C (lower panel) and treated with either DMSO, serum and growth factor deprivation (3% FCS), or 5 μM MG132 for up to 96 h. Media was changed on the samples at 72 h and replaced with fresh inhibitor, thus the 96 h samples represent any additional death from 72 h. Cells were harvested and an Annexin V assay performed. The data shown is the mean of three independent experiments $\pm$ S.E.M. (**b**) Effect of proteasome inhibition on p53 levels in KIM-2 cells. KIM-2 cells were grown to confluency and treated over a 24 h period with 5 μM MG132 and whole cell extracts were subsequently prepared. Ten μg protein was subjected to SDS–PAGE analysis and Western blotting followed using a monoclonal p53 antibody. The $-$ive control is represented by 10 μg protein from KIM-2 cells which had been differentiated prior to harvesting, and the +ive controls are KIM-2 cells treated with 10 J/cm^2 of UV irradiation. (**c**) Effect of proteasome inhibition on p53, MdM2, and Bax levels in KIM-2 cells. KIM-2 cells were grown to confluency and treated over a 24 h period with 1 μM Lactacystin. Whole cell extracts were subsequently prepared and 10 μg protein subjected to SDS–PAGE analysis. Western blotting was performed using a p53, MdM2 and Bax antibodies

compared to the p53 null cells (Figure 5a). Cell cycle analysis identified a G2/M block in both the cell types in contrast to cell death, which was observed only in the p53 wt cells. At higher concentrations this cell cycle blockage was accompanied with the appearance of a pre-G1 DNA peak in the wt p53 cells e.g. 3% in the control cells, which increased to 31% with 10 μM MG132 treatment. In contrast the p53 null cells remained blocked in G2/M with very little death observed e.g. 13% pre-G1 staining cells for 10 μM MG132 treatment (Table 3). These results are consistent with a requirement for p53 in the apoptotic response induced by proteasome inhibition.

To determine if the effects on proteasome inhibition were specific to KIM-2 mammary epithelial cells we examined proteasome blockage in a second mammary epithelial cell line, HC11. In contrast to the KIM-2 data, HC11 cells were not induced to undergo apoptosis with MG132 treatment. These cells were treated at concentrations of up to 100 μM for 48 h, and no cell death was observed. Additionally Lactacystin treatment also failed to induce apoptosis in HC11 cells. The apoptotic response induced in KIM-2 cells may have been specific to these cells and to address this question Jurkat T cells were treated with MG132 (Figure 5b). Results showed a very similar effect to that in mammary epithelial cells, the contrast being that the level of death was greatly enhanced in the Jurkat cells ($>$80%). Western analysis showed that MG132 was having the effect of blocking protein degradation in HC11 mammary epithelial cells despite the fact that no apoptosis was observed. Increased ubiquitinated proteins were observed in the samples treated with MG132 (Figure 5c). The results observed in HC11 may be explained due to a mutation in p53 in this cell line[16] and add further evidence for a role of p53 in this apoptotic pathway.

Discussion

The results of the present study demonstrate that compounds capable of interfering with proteasome function induce apoptosis. KIM-2 mammary epithelial cells were incubated with inhibitors of the chymotrypsin-like function of the proteasome as well as with unrelated protease inhibitors. All of the three inhibitors that were capable of inducing cell death, MG132, Lactacystin and Calpain II Inhibitor, have been attributed with a function of perturbing the 26S proteasome and its degradation pathway. Of these peptides MG132 and

Table 3 Induction of a G2/Mitosis arrest in ES cells by proteasome inhibition

	p53 wt ES cells				p53 null ES cells			
Treatment (24 h)	**Pre-G1**	**G1**	**S**	**G2/M**	**Pre-G1**	**G1**	**S**	**G2/M**
	($\pm$S.E.M.)				($\pm$S.E.M.)			
Control (DMSO)	3 (0.2)	30 (0.6)	36 (0.8)	28 (0.6)	4 (0.3)	35 (0.8)	28 (0.6)	29 (0.8)
10 μM MG132	31 (0.7)	10 (0.4)	21 (0.8)	30 (1.1)	13 (0.4)	13 (0.5)	4 (0.9)	68 (1.2)

ES cells (E14 and R72D27) were treated with either 5, 10 or 25 μM MG132 for 24 or 48 h. Cells were harvested and fixed in 70% Ethanol prior to staining with Propidium Iodide for flow cytometric analysis. The data shown are expressed as the proportion of cells in each phase of the cell cycle

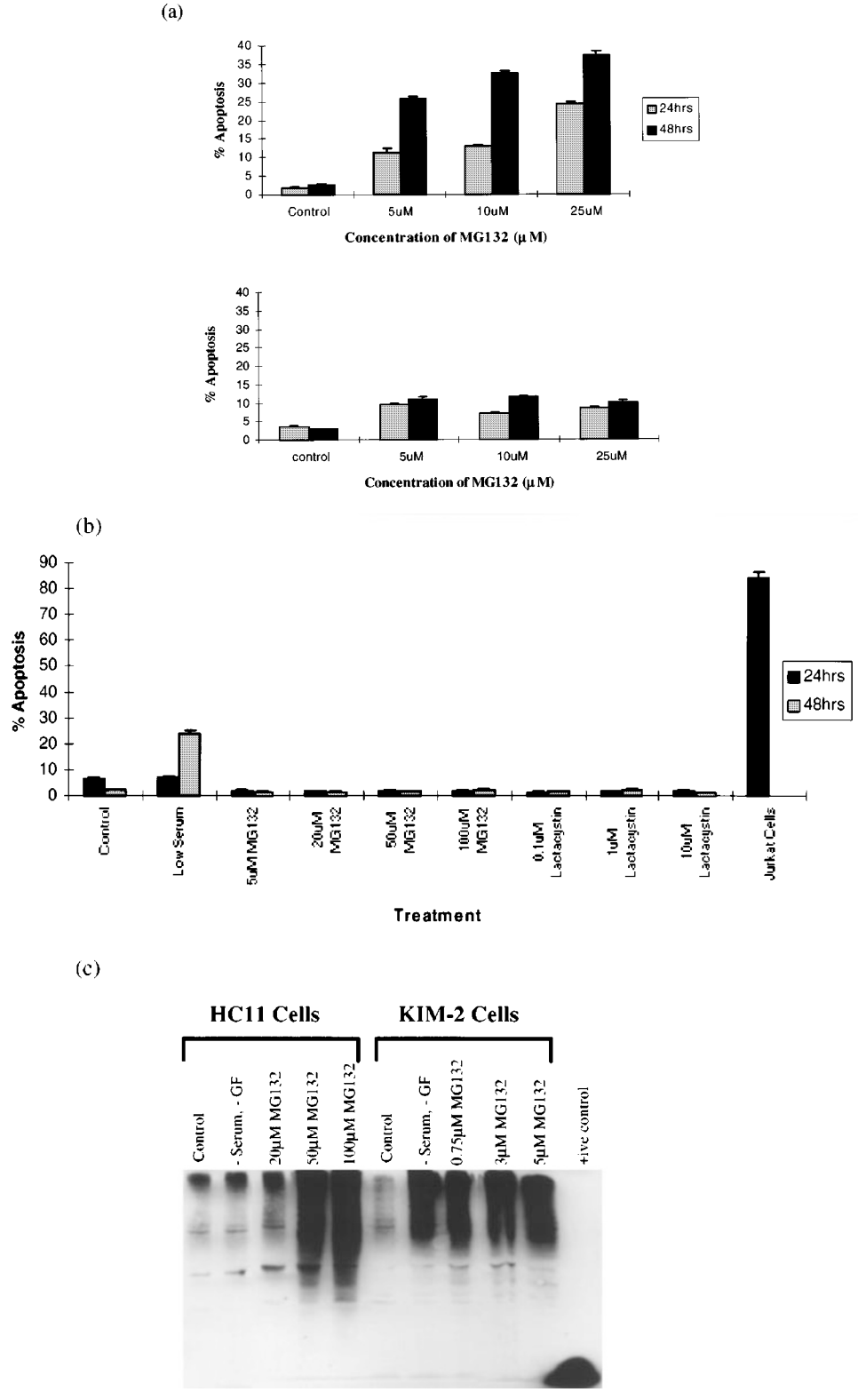

Figure 5 (**a**) p53 dependence on apoptosis induced by proteasome inhibition. ES cells containing wt p53 (E14 cells) (upper panel) and which were null for p53 (R72D27 cells) (lower panel) were treated with MG132 at 5, 10 and 25 μM for up to 48 h. Cells were harvested and an Annexin V assay performed. The results shown are expressed as the percentage of Annexin V positive cells, and represent the mean $\pm$ S.E.M. (**b**) HC11 mammary epithelial cells were harvested after 24 and 48 h treatment with a range of concentrations of MG132 and Lactacystin. Cells were then stained with acridine orange and subjected to a fluorescence microscopy where cell death was assessed. The data shown are means $\pm$ S.E.M. The hatched grey bar represent Jurkat T-cells treated with 2 μM MG132 for 24 h. (**c**) HC11 and KIM-2 cells were incubated with various concentrations of MG132 for 24 h prior to protein extraction. Whole cell extracts were analyzed by Western blot for ubiquitin-conjugated proteins using a DAKO polyclonal Ubiquitin antibody. Five μg purified ubiquitin (Sigma) was used as a positive control

Lactacystin are the more specific inhibitors of the 26S proteaseom[8] suggesting that the difference in response observed between these three inhibitors in this study is due to proteasome inhibition and not to calpain or cathepsin B inhibition. Given that MG132 had no effect on KIM-2 cells at 33°C, the effects observed at 37°C cannot be attributed to non-specific cytotoxicity. These results suggest that the proteasome degradation pathway is linked to the regulation of apoptotic cell death in mammary epithelia.

A major function of the 26S proteasome is the degradation of cell cycle proteins, i.e. it is responsible for the degradation of cyclins, cdks, cdk inhibitors, c-*myc*, p53, pRb, and E2F. All of the above proteins are involved in cell cycle regulation and have been reported to be actively degraded at specific points during the cell cycle. In some cases, their degradation is necessary to ensure correct progression through the cell cycle.[18] Therefore it seems plausible that if this pathway is perturbed then the major effects seen will be on the progression of the cell cycle. Our results agree with previous reports showing that in actively proliferating cells, proteasome function is essential for cell cycle progression.[11,19] Evidence to support this theory has come from studies in Rat1 cells, which had been rendered quiescent by serum starvation.[19] Proteasome inhibition failed to induce apoptosis in these quiescent cells. In contrast, both proliferating and differentiated PC12 cells could be induced to undergo apoptosis. Data conflicting with these results comes from work in neurons[10] and thymocytes,[9] which were seen to require proteasome function for the induction of an apoptotic response. These results can be explained by the non-proliferating status of these two cell types. Indeed, Grimm and co-workers[9] have shown that in thymocytes, long term exposure to proteasome inhibitors can induce a background level of cell death. Both the thymocyte and neuron experiments suggested that the proteasome acted upstream in the apoptotic response prior to the activation of caspases. Taken together, the data available would implicate the proteasome in functioning in both cell survival and cell death pathways. Whichever pathway the cell executes may depend on its proliferative state and other cell specific factors, which remain unknown to date.

The present study also describes a dependence on p53 function for the apoptotic response to proteasome inhibition. KIM-2 cells containing SV40 Tag at the semi-permissive temperature of 37°C underwent >30% apoptosis in response to proteasome inhibition, whereas at the permissive temperature of 33°C no induction of death was observed. Results from p53 null ES cells and HC11 cells, which are mutant for p53[16] confirmed this result. Although p53 presence was essential for apoptosis, no stabilisation of p53 was observed following proteasome inhibition. Examination of Bax and MdM2, p53-responsive genes, revealed no increase of these proteins following Lactacystin treatment suggesting that there was no p53-independent regulation of Bax and MdM2 expression in this system. This also dismissed stabilisation of Bax as a potential inducer of apoptosis in KIM-2 cells. Both KIM-2 mammary epithelial cells and ES cells when treated with MG132 appeared to have difficulty traversing cell cycle checkpoints, i.e. the G1/S and G2/M boundaries, although in KIM-2 cells the G1/S-phase block appeared to be more restricted by MG132 treatment. The evidence for cell cycle arrest by proteasome inhibition is currently conflicting. HeLa cells arrest in G2/M,[20] identical to CHO cells containing an ubiquitin activating enzyme mutation.[21] Yeast cells containing ubiquitin mutants also G2/M arrest,[22] whereas the human leukemic HL60 cells arrest in G1/S phase.[11] It is possible that a G1/S-phase arrest could be induced as a result of a p53 dependent apoptotic response. p53 is linked to the G1/S-phase checkpoint through a DNA damage pathway, but p53 can trigger apoptosis via other responses, i.e. in response to metabolic imbalance or to calcium phosphate[13] and can also induce death from other stages of the cell cycle i.e. from G2/M following DNA damage induced apoptosis.[23]

The difference in cell cycle arrest observed between the two cell lines used in this study and other published reports cannot be explained by the presence of Tag. SV40 Tag complexes with p53, and pRb at the permissive temperature of 33°C. p53 is a known target of the ubiquitin-proteasome pathway and thus should be stabilised following proteasome inhibition. We observed no net accumulation of p53 in KIM-2 cells in response to proteasome inhibition. This may suggest that p53 is not degraded by the proteasome in KIM-2 cells but the interpretation is complicated by the possibility that more than one intracellular pool of p53 exists within these cells cultured at 37°C. p53 complexed to Tag may be stabilised whilst the free p53 would be available to the degradation machinery. This may have resulted in the net effect of no observed changes in the levels of the p53 protein.

In KIM-2 cells there is a potential disruption of pRb function due to the presence of SV40 Tag. pRb when complexed to Tag is unable to function in regulating the activity of the transcription factor E2F-1. E2F proteins are stabilised by binding unphosphorylated Rb proteins. This interaction prevents E2F-1 degradation by the 26S proteasome, and thus the protein is stable at the point in the cell cycle, G1/S-phase transition, at which it is essential for regulating.[24] When pRb is complexed to Tag, there is a loss of regulation of E2F1 activity and stability. As a consequence proteasome inhibition should result in elevated levels of E2F-1, which have been reported to promote S-phase entry followed by apoptosis.[25] This consequence of proteasome inhibition may suggest that death would be induced from G1/S-phase block.

Any model proposed for apoptotic cell death induced by proteasome inhibition may be highly complex. It is well documented that the sequential expression and inactivation of a variety of gene products finitely controls the cell cycle. The 26S proteasome degradation pathway is intricately intertwined with the control mechanisms of the cell cycle. A disruption in either of these pathways will lead to the inappropriate expression of a control protein for a particular stage of the cell cycle. Should a number of cell cycle proteins be inappropriately expressed, as would be the case with proteasome inhibition, then a cell may simply choose to abort cell cycle progression and as a consequence be induced to undergo an apoptotic

response. Death from proteasome inhibition is most likely a consequence of conflicting signals given to the cell. These are signals which the cell was not programmed to encounter simultaneously. Aberrant expression of a number of cell cycle proteins, most of which are substrates of the 26S proteasome pathway, has been documented to induce apoptosis. To try to dissect the precise signal which induces the cell to enter an apoptotic pathway will be complex as the proteasome degrades many proteins that have essential functions. It is becoming more apparent that the mechanisms controlling the cell cycle and apoptosis are linked. This link may be the proteasome which, dependent on the cell type and the signal received, can determine whether a cell will proliferate or die.

Materials and Methods

Cell culture and reagents

Murine KIM-2 mammary epithelial cells were cultured in 1 : 1 Dulbecco's Modified Eagle Media (DMEM) and Ham's F12 Media supplemented with 10% bovine calf serum, 5 μg/ml Insulin, 10 ng/ml epidermal growth factor, and 5 μg/ml linoleic acid. HC11 mammary epithelial cells were cultured in RPMI 1640 and supplemented with the same constituents as KIM-2 cells with the exception that 20 ng/ml of epidermal growth factor was used. ES cells were cultured in BHK (Glasgow MEM) media supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5% foetal calf serum, 5% newborn calf serum, 0.1 mM β-mercaptoethanol, and murine LIF. To serum and growth factor deprive cells, KIM-2 cells were incubated in DMEM-F12 containing 3% calf serum, whereas HC11 cells were treated with RPMI 1640 alone.

Cbz-leucinyl-leucinyl-leucinal (MG132), Ac-Leucinyl-leucinyl-methional (ALLM), trans-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester (E64), leupeptin, pepstatin and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma. Lactacystin was purchased from Calbiochem. MG132 and Lactacystin were diluted in Me_2SO, ALLM was diluted in Ethanol, Pepstatin and PMSF were diluted in methanol, and the remainder of the compounds were water soluble.

Characterisation of apoptosis

Cells were treated with protease inhibitors or dilutant alone. Supernatant and monolayer cells were harvested by centrifugation and fixed in 70% ethanol in PBS for staining with acridine orange. Equal volumes of cells and acridine orange (5 mg/ml in PBS) were mixed on a microscope slide and examined by fluorescence microscopy. For annexin V analysis, cells were harvested by centrifugation and 10^5 cells were stained with annexin V and propidium iodide using an R&D Systems Apoptosis Detection Kit as per manufacturer's instructions. For cell cycle analysis, 1×10^6 fixed cells were rehydrated in PBS at room temperature for 10 min, followed by staining with propidium iodide (5 mg/ml). All samples were analyzed using a Coulter Epics XL flow cytometer.

Western blot analysis

Cell lysates (10 μg) were electrophoresed in SDS-polyacrylamide gels and transferred onto either nitro-cellulose or PVDF membranes. Primary antibodies against ubiquitin were purchased from DAKO, MdM2 (SMP-14) from Santa Cruz, Bax (Ab-1) from Calbiochem, and p53 (pAb240) was kindly received from Prof. David Lane (Dundee). Secondary antibodies and detection reagents (ECL) were purchased from Amersham.

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