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Inhibition versus induction of apoptosis by proteasome inhibitors depends on concentration

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Abstract

We previously established that NF-κB DNA binding activity is required for Sindbis Virus (SV)-induced apoptosis. To investigate whether SV induces nuclear translocation of NF-κB via the proteasomal degradation pathway, we utilized MG132, a peptide aldehyde inhibitor of the catalytic subunit of the proteasome. 20 μM MG132 completely abrogated SV-induced NF-κB nuclear activity at early time points after infection. Parallel measures of cell viability 48 h after SV infection revealed that 20 μM MG132 induced apoptosis in uninfected cells. In contrast, a lower concentration of MG132 (200 nM) resulted in partial inhibition of SV-induced nuclear NF-κB activity and inhibition of SV-induced apoptosis without inducing toxicity in uninfected cells. The specific proteasomal inhibitor, lactacystin, also inhibited SV-induced death. Taken together, these results suggest that the pro-apoptotic and anti-apoptotic functions of peptide aldehyde proteasome inhibitors such as MG-132 depend on the concentration of inhibitor utilized and expand the list of stimuli requiring proteasomal activation to induce apoptosis to include viruses.

Keywords: proteasome inhibitors; apoptosis; Sindbis Virus

Abbreviations: NAC, N-acetylcysteine; SV, Sindbis Virus; TNFα, tumor necrosis factor-α; IL-1, interleukin-1; PMA, phorbol 12-myristate 13-acetate; OA, okadaic acid; CLP I, calpain inhibitor I; CLP II, calpain inhibitor II; LPS, lipopolysaccharide

Introduction

Infection of a variety of cultured cells with Sindbis virus (SV), an alphavirus, will induce cellular changes characteristic of apoptosis, including chromosome condensation, DNA fragmentation and membrane blebbing (Levine *et al*, 1993; Lin *et al*, 1995). Additionally, the ability of SV to induce apoptosis *in vivo*, in neurons of newborn mice, has been correlated with its ability to induce a fatal encephalitis in these mice (Lewis *et al*, 1996; Ubol *et al*, 1994). As SV infection in mice has served as a model for viral encephalitis in humans (Strauss and Strauss, 1994), enhanced understanding of the mechanisms by which SV induces apoptosis may reveal novel therapeutic approaches to human encephalitides such as Eastern and Western equine encephalitis.

Previous studies from our laboratory have begun to elucidate some of the pathways by which SV induces apoptosis. We demonstrated that infection of cultured AT3 prostate carcinoma cells induces nuclear translocation (activation) of the transcription factor NF-κB, and that activation of NF-κB is required for SV-induced death in this cell line (Lin *et al*, 1995). Furthermore, we correlated the anti-apoptotic effects of the thiol antioxidant, N-acetylcysteine (NAC) and the anti-apoptotic protein, Bcl-2, with their ability to inhibit activation of NF-κB early after SV infection (Lin *et al*, 1995). Thus, additional information regarding the mechanisms by which SV induces NF-κB activation may not only provide important insights into viral pathogenesis but also the molecular targets of the pluripotent anti-apoptotic agents, NAC and Bcl-2.

Several recent studies have begun to define common pathways involved in NF-κB activation in response to a variety of inflammatory and pathologic stimuli (Alkalay *et al*, 1995a,b; Beg *et al*, 1993; Chen *et al*, 1995; DiDonato *et al*, 1995; Henkel *et al*, 1993; Palombella *et al*, 1994; Thanos and Maniatis, 1995; Traenckner, *et al*, 1994, 1995). The prototype of NF-κB is a heterodimeric complex composed of p50 (NF-κB 1) and RelA (p65) subunits (Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990). Under basal conditions, NF-κB is sequestered in the cytoplasm by its association with an inhibitory molecule, IκB (Baeuerle and Baltimore, 1988). Activation of NF-κB results from proteolysis of IκB. Recent data suggest that diverse stimuli, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), phorbol 12-myristate 13-acetate (PMA), okadaic acid (OA) and calyculin A, lead to phosphorylation of IκB at Serine-32 and/or Serine-36 (Brown *et al*, 1995; Chen *et al*, 1995; DiDonato *et al*, 1996; Traenckner *et al*, 1995). The phosphorylation of these residues in the N-terminal region of IκB is required for ubiquitination of IκB (Alkalay *et al*, 1995b; Chen *et al*, 1995; Sherer *et al*, 1995), as well as its subsequent proteolytic degradation by the 26S proteasome complex (Alkalay *et al*, 1995b; Palombella *et al*, 1994). Degradation of IκB unmasks a nuclear localization signal on the NF-κB complex (Beg and Baldwin, 1993) and allows free NF-κB to translocate to the nucleus where it can influence the expression of its target genes.

Despite growing evidence suggesting that activation of NF-κB by different stimuli is through a common proteasomal degradation pathway, it is not known whether the activation of NF-κB induced by infection with SV, occurs

through the same machinery. Indeed, recent studies have identified a novel pathway of NF-κB activation that involves tyrosine phosphorylation without proteasomal IκB degradation (Imbert *et al*, 1996). To distinguish among these possibilities, we chose peptide aldehyde proteasome inhibitors, which have been previously applied to cultured cells as competitive inhibitors of proteasomal function (Griscavage *et al*, 1996; Maggirwar *et al*, 1995; Palombella *et al*, 1994; Reed *et al*, 1995; Rock *et al*, 1994). Herein, we used these inhibitors to determine whether SV induces NF-κB activation by stimulating proteasomal degradation of I-κB and if so, whether proteasome inhibitors could also block SV-induced apoptosis.

Results

20 μM MG132 inhibits SV-induced NF-κB activation

MG132 has been shown to be a potent inhibitor of the chymotrypsin-like activity of the proteasome complex (Rock *et al*, 1994). Because other laboratories have previously utilized 20 μM MG132 to inhibit the nuclear translocation of NF-κB in response to TNF-α (Chen *et al*, 1995; Reed *et al*, 1995) and Tax, a transactivator encoded by human T cell leukemia virus type-1 (HTLV-1) (Maggirwar *et al*, 1995), we utilized this concentration to determine whether SV-induced NF-κB activation in AT3 cells could be inhibited. As shown in Figure 1A, 20 μM MG132 completely inhibits SV-induced NF-κB nuclear DNA binding activity at all time points examined.

To verify that the inhibition of SV-induced nuclear NF-κB binding activity by MG132 correlated with inhibition of IκB-α degradation in the cytoplasm, we examined IκB-α levels in cytoplasmic extracts derived from cells treated with and without MG132. In contrast to cytoplasmic extracts from cells infected with SV alone, reduced degradation of IκB-α (Figure 1B) was observed in SV-infected cells treated with MG132. These results are consistent with the notion that MG132 (20 μM) inhibits proteasomal degradation of IκB-α and thereby inhibits SV-induced NF-κB activation.

MG-132 can induce or inhibit apoptosis depending on the concentration utilized

In previous studies, we demonstrated that NF-κB is required for SV-induced apoptosis in AT3 cells (Lin *et al*, 1995). We therefore investigated whether MG132's ability to inhibit SV-induced NF-κB activation up to 6 h after infection could be correlated with its ability to inhibit SV-induced apoptosis 48 h after infection. Consistent with a previous report involving proteasome inhibitors applied to cells *in vitro* (Imajoh-Ohmi *et al*, 1995), we found that MG132 (20 μM) induced cell death with morphologic features of apoptosis in uninfected AT3 cells (data not shown). However, we found that a lower concentration of MG132 (200 nM), which has no effect on viability in uninfected AT3 cells, dramatically inhibits SV-induced apoptotic morphology (data not shown) and subsequent secondary necrosis (Figure 2A). Secondary necrosis of apoptotic cells is seen commonly *in vitro* possibly because of the absence of professional phagocytic cells in the culture dish.

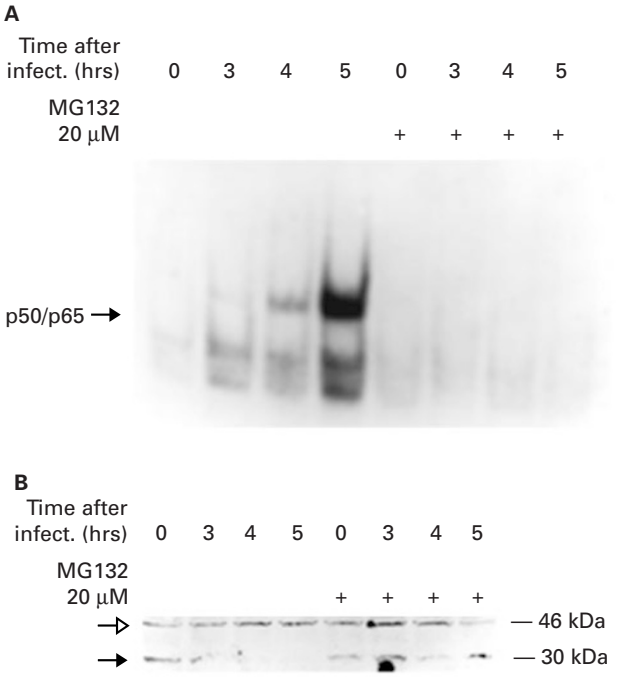

Figure 1 20 μM MG132 blocks SV-induced NF-κB activation and IκB-α degradation in AT3 cells. Nuclear and cytoplasmic extracts were harvested from SV infected AT3 cells at various time points in the absence or presence of 20 μM MG132. (**A**) EMSA was performed with 4–10 μg of nuclear extracts using a ^{32}P labeled oligonucleotide with a consensus NF-κB sequence from the MHC Class I promoter. Arrow points to predominant complex induced by SV, p50-p65. The composition of the induced NF-κB complexes was demonstrated in a previous study (26). (**B**) 20 μg cytoplasmic extracts were used in Western blot analysis against IκB-α. Black arrow points to IκB-α. White arrow points to non-specific protein

The concentrations of MG132 found to prevent SV-induced death were 100-fold lower than those used previously by us (Figure 1A) and other groups to inhibit stimulus dependent NF-κB activation. We therefore examined whether the protective effects of 200 nM MG132 could be correlated with inhibition of NF-κB activation in AT3 cells. A decrease in nuclear NF-κB DNA binding was observed at each time point after infection as measured by EMSA (Figure 2B). Altogether, these results suggest that the protective effects of 200 nM MG132 can be correlated with partial inhibition of nuclear activation of NF-κB.

To determine whether the protective effects of MG132 could be mimicked by other proteasome inhibitors, we examined the effects of the peptide aldehydes, calpain inhibitor I and calpain inhibitor II. Previous studies have shown that MG132, calpain inhibitor I and calpain inhibitor II can each inhibit proteasome-catalyzed hydrolysis of hydrophobic peptides in a test tube with the following rank order of potencies: MG132 (K_i=4.0 nM) $>$ calpain inhibitor I (K_i=140 nM) $>$ calpain inhibitor II (K_i=1 μM) (Joe *et al*, 1996). Viability studies in AT3 cells infected with SV in the presence of each of these peptide aldehyde inhibitors revealed 24 ± 1 (mean $\pm$ S.E.M., n=3) percent of cell death in the presence of 200 nM MG132, 33 ± 7% cell death in the presence of 20 μM calpain inhibitor I and 53 ± 5% cell

death in the presence of 20 μM calpain inhibitor II as compared to 67$\pm$4% cell death in non-treated, SV infected cells (Figures 2A and 3). Thus, the rank order of efficacy of MG132, calpain inhibitor I and calpain inhibitor II in preventing SV-induced death in intact cells agrees with their relative abilities to inhibit the hydrolysis of hydrophobic peptides, casein and ubiquitinated proteins, by the proteasome in the test tube.

We then determined whether the relative abilities of calpain inhibitor I and calpain inhibitor II to inhibit SV-induced death could be correlated with their effects on SV-induced NF-κB activation as measured by EMSA. Similar to MG132 (200 nM), the protective peptide aldehyde, calpain inhibitor I (20 μM), diminished SV induced NF-κB activation, whereas the ineffective calpain inhibitor II, had no effect (data not shown).

To determine whether the low concentrations of MG132 used in the present study also attenuate NF-κB activation in response to other stimuli, we examined the effect of MG132, calpain inhibitor I, and calpain inhibitor II on lipopolysaccharide (LPS) induced NF-κB activation in AT3 cells. As shown in Figure 4, concentrations of MG132 and calpain inhibitor I similar to those used to inhibit SV-induced

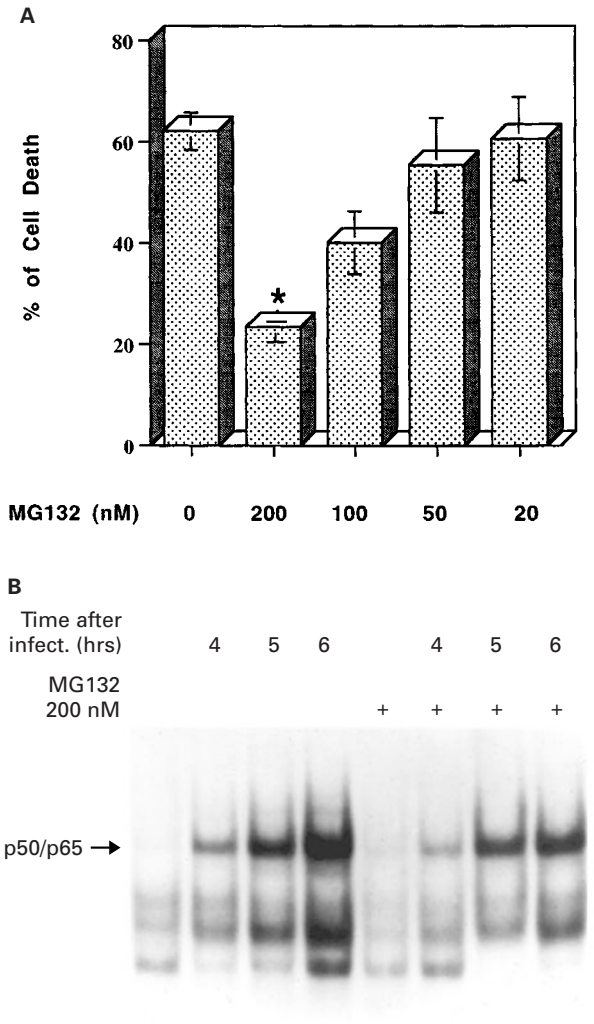

Figure 2 200 nM MG132 inhibits SV-induced apoptosis and NF-κB DNA binding activity in AT3 cells. (**A**) Concentration response of protection by MG132 48 h after SV infection in AT3 cells. Per cent of cell death was determined by the per cent LDH release into media. Results are expressed as mean$\pm$S.E.M. (n=3). *Statistical analysis revealed that viability in the presence of 200 nM MG132 was statistically different from control ($P<0.01$) and that there exists a linear trend in dose response (0, 20, 50, 100 and 200 nM MG132; $P<0.01$) (**B**) Effects of 200 nM MG132 on SV-induced NF-κB activation in AT3 cells. EMSA was performed using nuclear extracts from infected cells, 4, 5, or 6 h after infection in the absence or presence of 200 nM MG132. Arrow points to predominant complex induced by SV, p50-p65. In addition to the EMSA's shown we also performed immunoblots using an antibody against the p65 subunit of NF-κB and the same nuclear extracts used for EMSAs. The changes in the p65 levels in the nucleus correlated well with the gel shift results. Additionally, the p65 antibody recognized a non-specific band whose levels were similar in all treatment groups (data not shown). On the basis of these results, we conclude that the amount of protein added to each lane was the same and that distinct protein loading cannot account for the partial inhibition of NF-κB seen in the presence of 200 nM MG-132

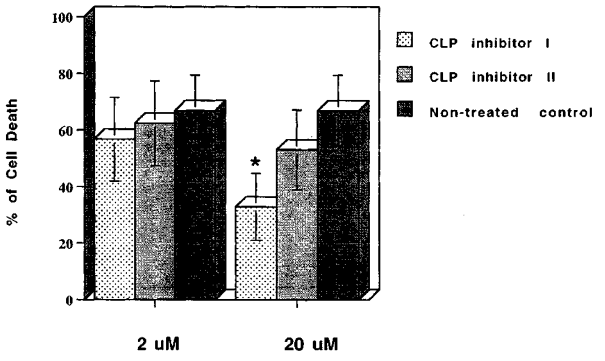

Figure 3 Effects of calpain inhibitor I (CLP inhibitor I) and calpain inhibitor II (CLP inhibitor II) on SV-induced apoptosis in AT3 cells. Concentration response of protection by calpain inhibitor I and calpain inhibitor II 48 h after SV infection in AT3 cells. Data were expressed as mean$\pm$S.E.M. (n=3) *Statistical analysis revealed that CLP I at 20 μM was different from control ($P<0.01$)

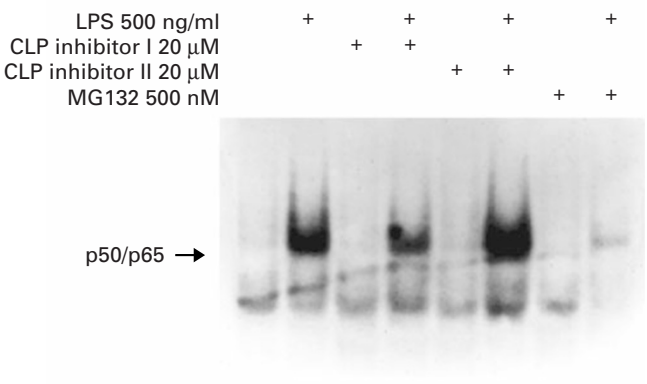

Figure 4 Effects of MG132, CLP I and CLP II on lipopolysaccharide (LPS)-induced NF-κB activation in AT3 cells. Nuclear extracts were performed after 30 min of LPS (500 ng/ml) treatment at the presence of 20 μM calpain inhibitor I, 20 μM calpain inhibitor II or 500 nM MG132. Arrow indicates the p50-p65 complex

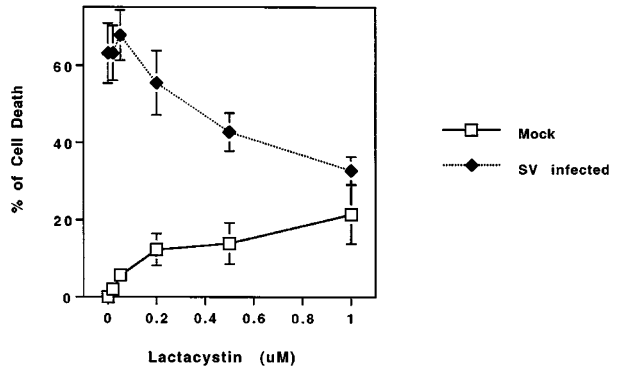

Figure 5 The more specific proteasome inhibitor, lactacystin abrogates SV-induced death. Concentration response of protection by lactacystin 48 h after SV infection in AT3 cells. Per cent of cell death for the SV-infected group was determined by dividing the cell viability in SV-infected cells plus lactacystin by the cell viability in cells treated with lactacystin alone and subtracting this value from 100. For the mock-infected group, percent of cell death was determined by dividing the cell viability in the mock infected group plus lactacystin by the cell viability in cells mock infected alone and subtracting this value from 100. Results are expressed as mean ± S.E.M. (n=3)

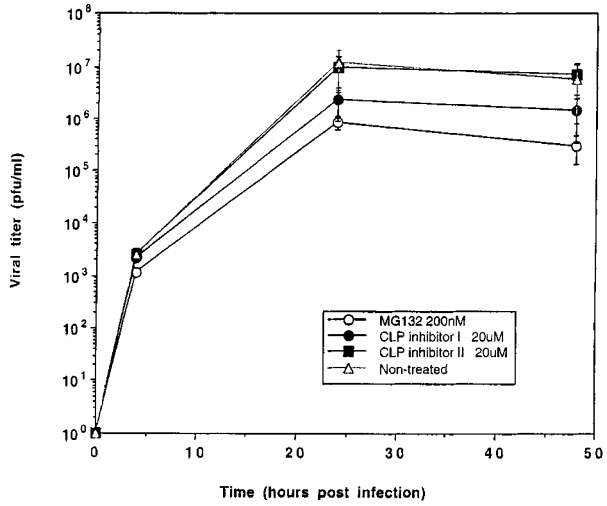

Figure 6 Effects of MG132, CLP inhibitor I and CLP inhibitor II on cumulative SV production in AT3 cells. At 4, 24 or 48 h after SV infection, the media of infected cells at the absence or presence of various peptide aldehyde were collected and viral titers were measured in plaque forming units per ml of media (PFU/ml). Error bars represent S.E.M. (n=3)

NF-κB activation and apoptosis, also inhibited LPS-induced NF-κB activation. Additionally, calpain inhibitor II, which had no effect on SV-induced NF-κB activation or apoptosis, also had no effect on LPS-induced NF-κB activation.

These observations suggest that the MG132 and calpain inhibitor I are acting in AT3 cells to inhibit proteasomal activation in response to SV infection, thereby attenuating SV-induced death. Consistent with this conclusion, we also found that the more specific, irreversible inhibitor of the proteasome, lactacystin (Fentenany *et al*, 1994) also inhibits SV-induced death. In contrast to reversible inhibitors of the proteasome, MG132 and calpain inhibitor I, the concentrations of lactacystin that were maximally protective also induced a small amount of cell death in uninfected cultures (Figure 5).

Cumulative SV production is altered by proteasome inhibitors

As previous studies have shown that the anti-apoptotic protein Bcl-2 not only inhibits SV-apoptosis but also inhibit SV replication, we examined whether proteasomal inhibitors influence the viral replication cycle. At each time point measured (Figure 6), concentrations of MG132 (200 nM) and calpain inhibitor I (20 μM) that were protective reduced the amount of virus released into the surrounding media, while the non-protective calpain inhibitor II had no effect. These results suggest that, like protection by overexpression of Bcl-2, protection by proteasomal inhibitors can be correlated with effects on the SV replication cycle.

Discussion

In this study, we report several primary observations. First, we establish that peptide aldehyde inhibitors of the proteasome inhibit virus-induced apoptosis. Our observations confirm and extend previous studies which have demonstrated that proteasome inhibitors block apoptosis in several non-viral paradigms (Cui *et al*, 1997; Delic *et al*, 1993; Grimm *et al*, 1996; Sadoul *et al*, 1996). A second central finding of our study is that the ability of proteasomal inhibitors to induce (Imajoh-Ohmi *et al*, 1995) as well as block apoptosis may be reconciled by the concentrations of drug used. For example, 20 μM MG132 induced apoptosis in uninfected AT3 cells (data not shown), whereas 200 nM MG132 abrogated apoptosis due to SV infection (Figure 2A). The low concentrations of MG132 utilized in the present study raised the possibility that this peptide aldehyde inhibitor may be acting on a molecular target distinct from the proteasome. However, several observations argue against this possibility.

First, the rank order of efficacy of MG132 and two other peptide aldehydes (calpain inhibitor I and calpain inhibitor II) in inhibiting proteasomal hydrolysis of peptide substrates in the test tube correlates with their abilities to block SV-induced apoptosis: MG132 $>$ calpain inhibitor I $>$ calpain inhibitor II.

Second, calpain inhibitor I and calpain inhibitor II inhibit cysteine proteases such as calpain and cathepsin B with similar efficacy in the test tube (Rock *et al*, 1994). These observations do not correlate with our results that calpain inhibitor I inhibits SV-induced apoptosis, but calpain inhibitor II does not and suggest that calpain and cathepsin B are not involved in SV-induced death.

Third, a more specific irreversible inhibitor of the proteasome, lactacystin, also prevents Sindbis Virus-induced death (Figure 5).

Finally, activation of NF-κB by many stimuli is known to result from proteasomal degradation of IκB. Herein, we demonstrate that activation of NF-κB in AT3 cells by SV or by another stimulus, LPS, is inhibited by similar concentra-

tions of MG132 and calpain inhibitor I used to inhibit SV-induced apoptosis in these cells (Figures 2B and 4). Additionally, calpain inhibitor II, which had no significant effect on SV-induced apoptosis, had no effect on SV (data not shown) or LPS-induced NF-κB activation in AT3 cells (Figure 4).

Along with previous observations from our laboratory that NF-κB DNA binding activity is required for SV-induced death in AT3 cells, the demonstration that the protective effects of proteasome inhibitors can be correlated with their abilities to inhibit SV-induced NF-κB activation in AT3 cells (Figure 2 and data not shown), is consistent with the notion that IκB is the critical substrate for proteasomal hydrolysis and activation of apoptosis in the present study. In support of this notion, a recent study provided a direct link between the proteasome dependent degradation of IκB-β, NF-κB translocation to the nucleus and the activation induced death of T cells that occurs through activation of the FasL gene and upregulation of the Fas gene (Cui *et al*, 1997).

An interesting question raised by the observations herein is, How can low and high concentrations of MG132 have opposite effects on SV-induced death and yet have similar, but not identical, effects on SV-induced NF-κB activity? It is established that peptide aldehydes such as MG132 act as competitive inhibitors of the proteasome (Rock *et al*, 1994) and that concentrations of MG132 required to inhibit degradation of one substrate may be different than the concentrations of MG132 required to inhibit the degradation of another substrate. We propose that high concentrations of MG132 (20 μM) stabilize pro-apoptotic proteins such as p53 (Gazos-Lopes *et al*, 1997) which leads to the induction of apoptosis despite the inhibition of NF-κB and in the absence of an apoptotic stimulus. In contrast, lower concentrations of MG132 (200 nM) do not influence p53 stability and thus do not affect the viability of mock-infected cells, but do incompletely affect the stability of SV related anti-apoptotic proteins such as IκB and thus lead to partial protection from cell death (see Figure 2).

The ability of inhibitors of the proteasome to alter the viral replication cycle (Figure 5) raises the possibility that a viral or cellular protein involved in SV replication might be effected by MG132 or calpain inhibitor I and the increased stability of this protein might account for the salutary effects of proteasome inhibitors. One candidate viral protein whose increased stability may influence the viral life cycle, is the SV RNA polymerase, nsP4 (de Groot *et al*, 1990). Previous studies have demonstrated that nsP4 is degraded by the N-end rule pathway, which is believed to be a ubiquitin-proteasome dependent pathway (de Groot *et al*, 1991; Varshavsky, 1992). While the importance of nsP4 degradation for SV replication has not been established, it has been postulated that SV has evolved to take advantage of the ubiquitin-proteasome pathway to regulate its life cycle. Indeed, a regulatory mechanism involving the ubiquitin-proteasome pathway may apply to a variety of viruses (Scheffner *et al*, 1990). In this regard, it is unlikely that inhibition of IκB degradation is responsible for the changes in SV replication observed as previous studies have shown that agents which inhibit IκB degradation and SV-induced apoptosis do not influence SV replication (Lin *et al*, 1995). Furthermore, previous studies have shown that prevention of SV (Levine *et al*, 1996) or influenza virus (Olsen *et al*, 1996) induced apoptosis by the pluripotent anti-apoptotic protein Bcl-2 is associated with inhibition of viral replication. Taken with these observations, our results are consistent with a model in which proteases such as the proteasome, required for virus-induced apoptosis, also positively regulate virus replication. Such a scheme would be adaptive for viruses, for it would maximize viral burden prior to the death of the host cell.

In addition to IκB-α and nsp4, another group of proteins known to be affected by proteasome inhibitors are cell cycle regulatory proteins. Indeed, several groups have shown that proteasome inhibitors block cell cycle progression and arrest cells in G_0/G_1 (Ciechanover, 1994; Fentenany *et al*, 1994). The possibility that proteasome inhibitors block cell cycle progression and prevent SV-induced death is interesting in light of recent studies which have implicated proliferation pathways in the execution of Sindbis Virus as well as growth factor deprivation induced death (Ferrari and Greene, 1994; Joe *et al*, 1996).

In summary, we demonstrate that inhibitors of the proteasome abrogate apoptosis induced by SV in AT3 cells. These observations are congruent with previous observations demonstrating a requirement for the proteasomal activation in thymocyte as well as neuronal apoptosis (Delic *et al*, 1993; Grimm *et al*, 1996; Sadoul *et al*, 1996; Cui *et al*, 1997). Although the precise target of proteasomal activity which leads to cell death remains unclear, our study, in concert with previous studies (Delic *et al*, 1993; Grimm *et al*, 1996; Sadoul *et al*, 1996; Cui *et al*, 1997), expands the list of proteases involved in apoptosis to include not only ICE family proteases (Miura *et al*, 1993) and granzymes (Shresta *et al*, 1995), but the proteasome complex as well.

Materials and Methods

Materials

MG132 (carbobenzoxyl-leucinyl-leucynyl-leucinal-H, CbZ-LLL) was generously provided by Proscript (Cambridge, MA). Calpain inhibitor I (N-acetylleucinyl-leucinyl-norleucinal, Ac-LLnL-CHO) and calpain inhibitor II (N-acetyl-leucinyl-leucinyl-normethioninal, Ac-LLM-CHO) were provided by Dr Edwin George. Lipopolysaccharide (LPS) was from Sigma. Lactacystin was obtained from Calbiochem. Rabbit polyclonal antibodies against IκB-α, were from Santa Cruz Biotechnology.

Cell culture and viability studies

AT3 cells were grown as previously described (Lin *et al*, 1995). For viability studies, 10^5 cells were plated per well in 12 well plates. One day after plating, the media was replaced with fresh media containing protease inhibitors. After pretreatment for 1 h with protease inhibitors, SV (strain AR339) was added to the media at a multiplicity of infection (m.o.i.) of 5–10 plaque forming units (PFU) per cell. Viability was assessed 48 h later by assay of lactate dehydrogenase release (Ratan *et al*, 1994) or by MTT assay. Single cell assessment of apoptosis was carried out using the DNA intercalating dye, propdidium iodide (10 μg/

ml) and fluorescence microscopy as previously described (Ratan *et al*, 1994).

Electrophoretic mobility shift assay (EMSA)

4×10^6 AT3 cells were infected with SV (m.o.i.=5), and cells were trypsinized and pelleted 3, 4, 5 or 6 h after infection. The subsequent extraction steps were as previously described (Lin *et al*, 1995). Supernatants from the first spin (cytoplasmic extracts) were aliquoted and stored at -70°C for analysis of I-κB α by Western blotting. Supernatants from the second spin (nuclear extracts) were used for EMSAs as previously described (Lin *et al*, 1995). Aliquoted cytoplasmic and nuclear extracts were precipitated using 3% perchloric acid and the protein concentration of each extract was measured by the bicinchoninic acid method (Pierce).

Immunoblot analysis

20 μg of protein from cytoplasmic (for IκB-α) extracts were boiled in Laemmli buffer and electrophoresed under reducing conditions on 12% polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a mini trans-blot cell apparatus at 20 V overnight at 4°C. Non-specific binding was inhibited by incubation in Tris-buffered saline (TBST: 50 mM Tris-HCl, pH 8.0, 0.9% NaCl, 0.1% Tween-20) containing 5% non-fat dried milk for 2 h. Anti-IκB-α antibody was diluted in 5% milk-TBST at 1 : 100 dilution. Membranes were then exposed to primary antibodies for 2 h followed by extensive washing with TBST. Following exposure of membranes to horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Amersham, 1 : 5000 in TBST) for 1 h, immunoreactive proteins were detected according to the enhanced chemiluminescent protocol (DuPont NEN).

Replication *in vitro*

AT3 cells were cultured and infected as described above. Infectious medium was collected from three wells at 4, 24 and 48 h post infection in the presence of various peptide aldehydes. Virus titers were quantified by plaque formation in BHK-21 (baby hamster kidney) cells (PFU/ml) (Lin *et al*, 1995).

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References

Alkalay I, Yaron A, Hatzubai A, Jung S, Avraham A, Gerlitz O, Pashut-Lavon I and Ben-Neriah Y (1995a) In vivo stimulation of IκB phosphorylation is not sufficient to activate NF-κB. Mol. Cell. Biol. 15: 1294 – 1301

Alkalay I, Yaron A, Hatzubai A, Orian A, Ciechanover A and Ben-Neriah Y (1995b) Stimulation-dependent IκB-α phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA 92: 10599 – 10603

Baeuerle PA and Baltimore D (1988) IκB: a specific inhibitor of the NF-κB transcription factor. Science 242: 540 – 546

Beg AA and Baldwin Jr, AS (1993) The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. 7: 2064 – 2070

Beg AA, Finco TS, Nantermet PV and Baldwin Jr, AS (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκB-α: a mechanism for NF-κB activation. Mol. Cell. Biol. 13: 3301 – 3310

Brown K, Gerstberger S, Carlson L, Franzoso G and Siebenlist U (1995) Control of IκB-α proteolysis by site-specific signal-induced phosphorylation. Science 267: 1485 – 1488

Chen Z, Hagler J, Palombella VJ, Melandri F, Sherer D, Ballard D and Maniatis T (1995) Signal-induced site-specific phosphorylation targets IκB-α to the ubiquitin-proteasome pathway. Genes Dev. 9: 1586 – 1597

Ciechanover A (1994) The ubiquitin-proteasome proteolytic pathway. Cell 79: 13 – 21

Cui H, Matsui K, Omura S, Schauer SL, Matulka RA, Sonenshein GE and Ju S-T (1997) Proteasome regulation of activation-induced T cell death. Proc. Natl. Acad. Sci., USA 94: 7515 – 7520

deGroot RJ, Hardy WR, Shirako Y and Strauss JH (1990) Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteins. Evidence for temporal regulation of polyprotein processing in vivo. EMBO J. 9: 2631 – 2638

deGroot RJ, Rümenapf T, Kuhn RJ, Strauss E G and Strauss JH (1991) Sindbis virus RNA polymerase is degraded by the N-end rule pathway. Proc. Natl. Acad. Sci. USA 88: 8967 – 8971

Delic J, Morange M and Magdelenat H (1993) Ubiquitin pathway involvement in human lymphocyte gamma-irradiation-induced apoptosis. Mol. Cell. Biol. 13: 4875 – 4883

DiDonato JA, Mercurio F and Karin M (1995) Phosphorylation of IκB-α precedes but is not sufficient for its dissociation from NF-κB. Mol. Cell. Biol. 15: 1302 – 1311

DiDonato JA, Mercurio F, Rosette C, Jian W-L, Suyang H, Ghosh S and Karin M (1996) Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Mol. Cell. Biol. 16: 1295 – 1304

Fentenany G, Standaert RF, Reichard GA, Corey EJ and Schreiber SL (1994) A β-lactone related to lactacystin induces neurite outgrowth in a neuroblastoma cell line and inhibits cell cycle progression in an osteosarcoma cell line. Proc. Natl. Acad. Sci. USA 91: 3358 – 3362.

Ferrari G and Green LA (1994) Proliferative inhibition by dominant - negative ras rescues naive and neuronally differentiated PC12 cells from apoptotic death. EMBO J. 13: 5922 – 5928.

Gazos-Lopes U, Erhardt P, Yao R and Cooper GM (1997) p53-dependent induction of apoptosis by proteasome inhibitors. J. Biol. Chem. 272: 12893 – 12896

Ghosh S and Baltimore D (1990) Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) 344: 678 – 682

Grimm LM, Goldberg AL, Poirier GG, Schwartz LM and Osborne BA (1996) Proteasomes play an essential role in thymocyte apoptosis. EMBO J. 15: 3835 – 3844

Griscavage JM, Wilk S and Ignarro LJ (1996) Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-κB. Proc. Natl. Acad. Sci. USA 93: 3308 – 3312

Henkel T, Machleidt T, Alkalay I, Krönke M, Ben-Neriah Y and Baeuerle PA (1993) Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature (London) 365: 182 – 185

Imajoh-Ohmi S, Kawaguchi T, Sugiyama S, Tanaka K, Õmura S and Kikuchi H (1995) Lactacystin, a specific inhibitor of the proteasome, induces apoptosis in human monoblast U937 cells. Biochem. Biophys. Res. Commun. 217: 1070 – 1077

Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-dieckmann C, Farahifar D, Rossi B, Auberger PA and Peyron JF (1996) Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α. Cell 86: 787 – 798

Joe AK, Ferrari G, Jiang HH, Liang XH and Levine B (1996) Dominant inhibitory ras delays Sindbis virus-induced apoptosis in neuronal cells. J. Virol. 70: 7744 – 7751

Levine B, Huang Q, Issacs JT, Reed JC, Griffin DE and Hardwick JM (1993) Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. Nature (London) 361: 739 – 742

Levine B, Goldman JE, Jiang HH, Griffin DE and Hardwick JM (1996) Bcl-2 protects mice against fatal alphavirus encephalitis. Proc. Natl. Acad. Sci. USA 93: 4810 – 4815

Lewis J, Wesselingh SL, Griffin DE and Hardwick JM (1996) Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. J. Viol. 70: 1828 – 1835

Lin K-I, Lee S-H, Narayanan R, Baraban JM, Hardwick JM and Ratan RR. (1995) Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. J. Cell Biol. 131: 1149 – 1161

Maggirwar SB, Harhaj E and Sun S-C (1995) Activation of NF-κB/Rel by Tax involves degradation of IκB-α and is blocked by a proteasome inhibitor. Oncogene 11: 993 – 998

Miura M, Zhu H, Rotello R, Hartwieg EA and Yuan J (1993) Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homologue of the C. elegans cell death gene Ced-3. Cell 75: 653 – 660

Olsen CW, Kehren JC, Dybdahl-Sissoko NR and Hinshaw VS (1996) *bcl-2* alters Influenza virus yield, spread, and hemagglutinin glycosylation. J. Virol. 70: 663 – 666

Palombella VJ, Rando OJ, Goldberg AL and Maniatis T (1994) The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell 78: 773 – 785

Ratan RR, Murphy TM and Baraban JM (1994) Oxidative stress induces apoptosis in embryonic cortical neurons. J. Neurochem. 62: 376 – 379

Reed MA, Neish AS, Luscinskas FW, Palombella VJ, Maniatis T and Collins T (1995) The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. Immunity 2: 493 – 506

Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D and Goldberg AL (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78: 761 – 771

Sadoul R, Fernanedez P-A, Ouiquerez A-L, Martinou I, Maki M, Schroter M, Becherer JD, Irmler M, Tschopp J and Martinou J-C (1996) Involvement of the proteasome in the programmed cell death of NGF-deprived sympathetic neurons. EMBO J. 15: 3845 – 3852

Scheffner M, Werness BA, Huibregtse JM, Levine AJ and Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63: 1129 – 1136

Sherer DC, Brockman JA, Chen Z, Maniatis T and Ballard DW (1995) Signal-induced degradation of IκB-α requires site-specific ubiquitination. Proc. Natl. Acad. Sci. USA 92: 11259 – 11263

Shresta S, Heusel JW, Macivor DM, Wesselschmidt RL, Russell JH and Ley TJ (1995) Granzyme B plays a critical role in cytotoxic lymphocyte-induced apoptosis. Immunol. Rev. 146: 211 – 221

Strauss JH and Strauss EG (1994) The alphaviruses: Gene expression, replication, and evolution. Microbiol. Rev. 58: 491 – 562

Thanos D and Maniatis T (1995) NF-κB: A lesson in family values. Cell 80: 529 – 532

Traenckner EB-M, Wilk S and Baeuerle PA (1994) A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NF-κB. EMBO J. 13: 5433 – 5441

Traenckner EB-M, Pahl HL, Henkel T, Schmidt KN, Wilk S and Baeuerle PA (1995) Phosphorylation of human IκB-α on serine 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J. 14: 2876 – 1883

Ubol S, Tucker PC, Griffin DE and Hardwick JM (1994) Neurovirulent strains of alphavirus induce apoptosis in bcl-2 expressing cells: role of a single amino acid change in the E2 glycoprotein. Proc. Natl. Acad. Sci. USA 91: 5202 – 5206

Varshavsky A (1992) The N-end rule. Cell 69: 725 – 735