Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells

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Abstract The proteasome inhibitors lactacystin and AcLLNal induced p53-independent apoptosis in two human glioma cell lines, and the apoptosis was accompanied by up-regulation of immunoreactive wild-type p53, $p21^{Waf1}$, Mdm2, and $p27^{Kip1}$. Pretreatment with cycloheximide decreased the induction of cell death independently of p53 protein status, suggesting that the up-regulation of short-lived proteins is associated with proteasome inhibitor-induced apoptosis. Caspase-3-like proteases were activated in the proteasome inhibitor-mediated apoptosis, and the induction of cell death was inhibited more effectively in the presence of z-VAD.fmk than in the presence of Ac-DEVD.fmk, suggesting that caspases other than caspase-3 are involved. Nonetheless, there were no significant alterations in levels of immunoreactive Bcl-2, Bcl-x_L, Bax, Bad, and Bak, nor any evidence of cytochrome *c* release into cytosol and dissipation of $\Delta\Psi_m$. Thus, the proteasome inhibitor-induced apoptosis is mediated by a mitochondria-independent mechanism, and the once activated caspase-3 does not cause the cytochrome *c* release and the $\Delta\Psi_m$ disruption.

Key words: Lactacystin; AcLLNal; Caspase-3; Cytochrome *c*; Mitochondrial membrane potential; Apoptosis; Glioma

1. Introduction

The caspase family is implicated as a central component of the proteolytic machinery during apoptotic cell death [1]. In contrast, much less attention has been paid so far to the potential role of proteasomes in apoptosis. In higher eukaryotic cells, the proteasome is involved in the ATP/ubiquitin-dependent proteolysis of most of the nuclear and cytosolic proteins and in particular of short-lived proteins critical for cell proliferation and cell cycle regulation. Examples include the tumor suppressor protein p53 [2–4], various cyclins [5], and cyclin-dependent kinase inhibitor $p27^{Kip1}$ [6,7]. The natural product lactacystin, which specifically inhibits proteolytic activities of the proteasome [8], has been shown to induce apoptotic cell death in human monoblast U937 cells [9], and peptide aldehyde inhibitors of proteasome AcLLNal have also been shown to induce apoptosis in human prostate adenocarcinoma [10] and U937 cells [11]. In addition, other proteasome inhibitors cause apoptosis in human T-cell leukemia MOLT-4 cells [12], human leukemic HL60 cells [13], Rat-1 cells [14], and PC12 cells [14]. Nevertheless, proteasome inhibitors such as lactacystin and MG132 have been reported to protect thymocytes, T cell hybridomas, and PC12 cells against a number of different apoptosis inducers [15–18], and non-proliferating Rat-1 cells were no longer susceptible to apoptosis induced by proteasome inhibitors [14]. In addition, certain forms of apoptosis in quiescent cells such as lymphocytes [19] and thymocytes [15], or terminally differentiated cells such as sympathetic neurons [16], depended on a functional proteasome. However, neurons and thymocytes did not differ from other cells in being susceptible to apoptosis when persistently exposed to proteasome inhibitors [15,16], and inhibition of the proteasome still induced apoptosis of non-proliferating PC12 cells that had been induced to differentiate by treatment with nerve growth factor [14]. These results indicate that cell proliferation is one but not the only determinant of the cellular response to inhibition of the proteasome. It appears that the proteasome can function to promote either cell survival or death, depending on both proliferative state and cell type-specific factors.

The specifics of the proteolytic cascades may vary depending on cell type and stimulus used to trigger apoptosis. Proteasome inhibitor-induced apoptosis is reported to be associated with activation of caspase-3 [13,20]. The major mechanism for activating caspases is related to some of the changes that occur in mitochondria during apoptosis [21]. Another mechanism for initiating the proteolytic cascade is induced by ligation of TNF receptors, Fas/APO-1/CD95 [22,23], or granzyme B [24,25], and is directly coupled to caspase activation without any evidence of involvement of mitochondria. Our data show that proteasome inhibitor activates caspase-3-like proteases without any evidence of release of cyt *c* from mitochondria and collapse of $\Delta\Psi_m$, suggesting that proteasome inhibitor-induced apoptosis is associated with mitochondria-independent activation of caspase-3.

2. Materials and methods

2.1. Glioma cell culture

Two human glioma cell lines, wild-type p53-positive U-87MG [26]

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Abbreviations: AcLLNal, acetyl-leucinyl-leucinyl-norleucinal; $\Delta\Psi_m$, mitochondrial membrane potential; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's minimum essential medium; FCS, fetal calf serum; EDTA, ethylenediaminetetraacetic acid; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis(β-amino-ethylether)*N*,*N*′-tetraacetic acid; DTT, dithiothreitol; Cyt *c*, cytochrome *c*; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; mCICCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMA, phorbol myristate acetate

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and mutant p53-positive T98G cells [27], were obtained from American Type Culture Collection (Rockville, MD, USA), and were maintained in DMEM supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were exposed to 50 μM lactacystin or 50 μM AcLLNal (Cosmo Bio Inc., Tokyo, Japan) for the indicated times, and were stained with 2.5 μg/ml Hoechst 33258 after fixation with 4% paraformaldehyde. Apoptotic cells were assessed based on the morphological features characteristic of apoptosis and electrophoretic patterns of oligonucleosomal DNA laddering, and the viability of the cells was confirmed by the trypan blue dye exclusion method.

2.2. Determination of DNA fragmentation

DNA was prepared from cells as described previously [28]. The resulting DNA preparation was analyzed by 1.5% agarose gel electrophoresis in TBE buffer (89 mM Tris-borate buffer, pH 8.0/89 mM boric acid/22 mM EDTA) containing 0.5 μg/ml of ethidium bromide at 100 V/cm for 30 min.

2.3. Immunoblot analysis

Cells were lysed in a lysis buffer (20 mM Tris-HCl at pH 7.4/150 mM NaCl/0.5% NP-40/1 mM EDTA/50 μg/ml leupeptin/30 μg/ml aprotinin/1 mM PMSF), and then centrifuged at 15 000×*g* for 10 min. Equivalent amounts of cell lysates were heated in 2×loading buffer at 95°C for 3 min, then electrophoresed in 12% SDS-polyacrylamide gel, except for immunoblotting of PARP in which 8% SDS-polyacrylamide gel was used, and transferred onto PVDF membranes. The membranes were incubated with primary antibody against both wild-type and mutant p53 (Oncogene Science, Cambridge, MA, USA), $p21^{Waf1}$ (Oncogene Science), Mdm2 (Santa Cruz, Santa Cruz, CA, USA), $p27^{Kip1}$ (Santa Cruz), Bcl-2 (Pharmingen, San Diego, CA, USA), Bcl-x (Transduction, Lexington, KY, USA), Bax (Mobio, Nagoya, Japan), Bad (Transduction), Bak (Santa Cruz), p10 subunit of caspase-1 (Santa Cruz), p12 subunit of caspase-3 (Santa Cruz), PARP (a kind gift from Dr. Guy G. Poirier, CHUL Research Center, Quebec, Que., Canada), cyt *c* (Pharmingen), and cytochrome oxidase (Molecular Probes, Eugene, OR, USA), washed, and blotted with species-specific biotinylated secondary antibodies (Vector, Burlingame, CA, USA) and then with HRP-streptavidin (Vector). The membrane was then developed in ECL reagent (Amersham, Arlington Heights, IL, USA) and exposed to X-ray film.

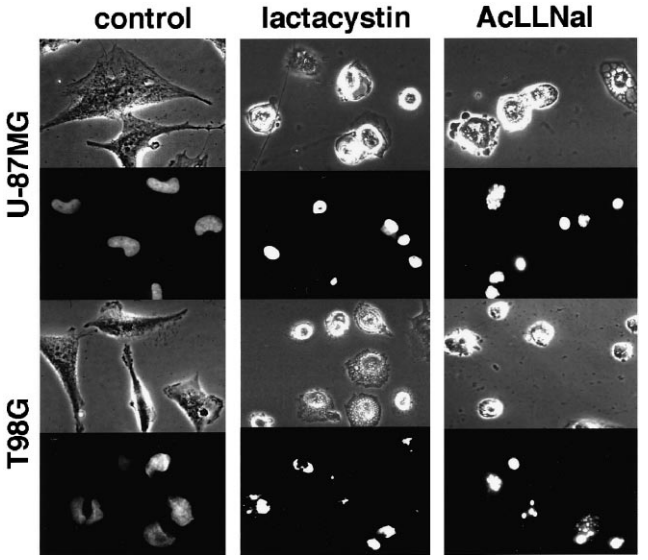

Fig. 1. Photomicrographs showing representative apoptotic morphological features induced by lactacystin or AcLLNal in two human glioma cell lines (U-87MG and T98G): cell shrinkage and surface blebbing by phase contrast images (lines 1 and 3) as well as nuclear condensation and fragmentation by Hoechst 33258 stain (lines 2 and 4). U-87MG cells were exposed to 50 μM lactacystin or 50 μM AcLLNal for 48 h, while T98G cells were exposed to 50 μM lactacystin or 50 μM AcLLNal for 24 h. Magnification ×86.

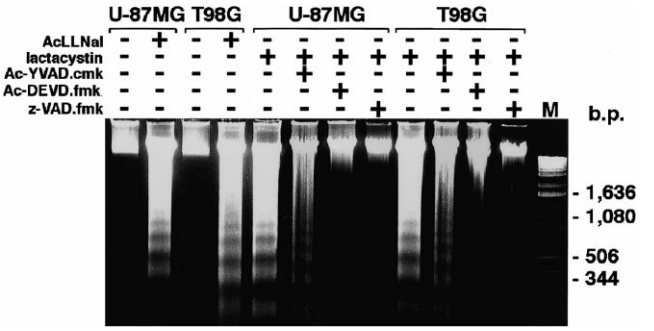

Fig. 2. Electrophoresis showing the induction of oligonucleosomal DNA laddering by AcLLNal treatment for 40 h in U-87MG cells (lane 2) and for 18 h in T98G cells (lane 4), or by lactacystin treatment for 30 h in U-87MG (lane 5) and for 15 h in T98G (lane 9). The induction of DNA laddering by lactacystin is not blocked in the presence of 100 μM Ac-YVAD.cmk (lanes 6 and 10) but is completely blocked in the presence of 100 μM Ac-DEVD.fmk (lanes 7 and 11) or 100 μM z-VAD.fmk (lanes 8 and 12). The formation of oligonucleosomal DNA fragments was determined by agarose gel electrophoresis. The results are from a representative study performed three times with comparable outcomes. Lanes 1 and 3, control; lane M, DNA size markers.

2.4. Cytochrome c release into cytosol

Cells were incubated in buffer A (20 mM HEPES, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/1 mM DTT/0.1 mM PMSF) containing 250 mM sucrose and homogenized with 10 strokes of a Teflon homogenizer. After centrifugation of homogenates twice at 750×*g* for 10 min at 4°C, the supernatants were centrifuged at 10 000×*g* for 15 min at 4°C. The resulting supernatants and pellets were frozen at −80°C as S10 cytosolic and mitochondrial fractions, respectively. In addition, the supernatants of the 10 000×*g* spin were further centrifuged at 100 000×*g* for 1 h at 4°C, and the resulting supernatants were also frozen at −80°C as S100 cytosolic fraction. Cyt *c* in mitochondrial and cytosolic fractions was immunologically probed with anti-cyt *c* monoclonal antibody, and contamination from mitochondria was examined by immunoblotting of cytochrome oxidase.

2.5. Measurement of mitochondrial membrane potential

Cells were incubated for 15 min in cultured medium at 37°C with 50 nM $DiOC_6(3)$ (Molecular Probes, Inc.) for measurement of the inner mitochondrial membrane potential ($\Delta\Psi_m$). As a positive control for $\Delta\Psi_m$ loss, cells were incubated at the same time with the uncoupling agent mCICCP (50 μM), a protonophore, which disrupts $\Delta\Psi_m$. Cells were washed once with PBS, collected by centrifugation at 1000×*g*, and stored in the dark at 4°C prior to analysis by flow cytometry using excitation of a single 488 nm argon laser. Data acquisition and analysis were performed using the CellFit software. Forward scatter, perpendicular scatter and $DiOC_6(3)$ fluorescence were measured with a 530/15 nm band pass filter. At least 10 000 events were collected per sample, with $DiOC_6(3)$ fluorescence recorded as logarithmic amplified data.

3. Results and discussion

3.1. Proteasome inhibitors induce apoptosis in glioma cells

To determine adequate concentrations of lactacystin or AcLLNal to induce glioma cell death, 10–100 μM lactacystin or AcLLNal were given for 72 h. The induction of the cell death was dose-dependent, and 50 μM lactacystin or 50 μM AcLLNal was chosen in the present experiment (cell viability during treatment with lactacystin or AcLLNal is shown in Fig. 5). During the treatment with lactacystin or AcLLNal, both U-87MG and T98G cells gradually showed apoptotic morphological features (Fig. 1): cell shrinkage and surface blebbing by phase contrast images as well as nuclear con-

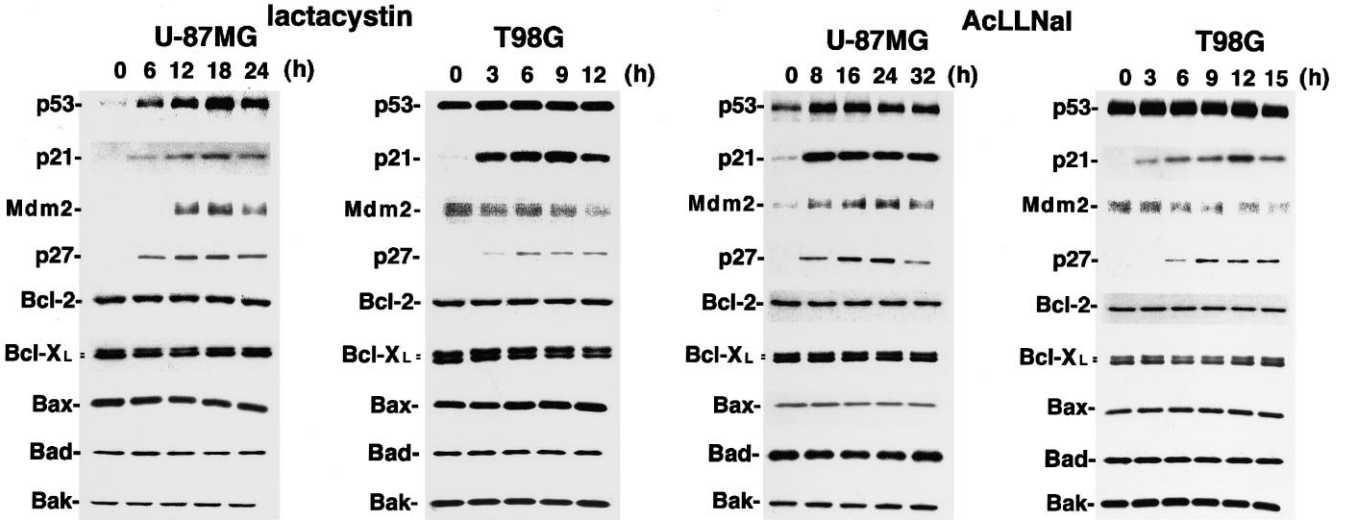

Fig. 3. Immunoblot analysis showing protein levels of p53, $p21^{Waf1}$, Mdm2, $p27^{Kip1}$, Bcl-2, Bcl-x_L, Bax, Bad, and Bak. Human glioma cells (U-87MG and T98G) were exposed to 50 μM lactacystin for 24 h in U-87MG and for 12 h in T98G, or to 50 μM AcLLNal for32 h in U-87MG and for 15 h in T98G, and steady-state levels of p53, $p21^{Waf1}$, Mdm2, $p27^{Kip1}$, Bcl-2 Bcl-x_L, Bax, Bad, and Bak were monitored by immunoblot analysis. The results are from a representative study performed three times with comparable outcomes.

densation and fragmentation by Hoechst 33258 stain. In addition, unambiguous electrophoretic patterns of oligonucleosomal DNA laddering were observed in both U-87MG and T98G cells (Fig. 2), indicating that lactacystin and AcLLNal induce apoptotic cell death. AcLLNal is also a better inhibitor of calpain than any of the catalytic activities of the proteasome, and complete inhibition of calpain is obtained with 10 μM AcLLNal [29]. However, 0.1–10 μM calpeptin, a specific inhibitor of calpain, which was enough high in concentration to inhibit the activity of calpain [30], did not induce apoptosis in the present glioma cell lines (unpublished data). In addition, the concentration of AcLLNal used in the present study is comparable to that required for induction of apoptosis in human adenocarcinoma cells [10] and U937 cells [11]. It is suggested, therefore, that the AcLLNal-induced apoptosis is due to the inhibition of proteasome but not calpain.

3.2. *Inhibition of proteasomal activity is accompanied by accumulation of p53, $p21^{Waf1}$, Mdm2, and $p27^{Kip1}$*

Since the anti-human p53 antibody used in the present study recognizes both wild-type and mutant p53 [31], p53 visualized by the present immunoblot analysis indicates wild-type in U-87MG cells and mutant in T98G cells. Immunoreactive wild-type p53, cyclin-dependent kinase inhibitor $p21^{Waf1}$, and p53 negative regulator Mdm2 in U-87MG cells were simultaneously up-regulated at least as early as 6 h after treatment with lactacystin or as early as 8 h following treatment with AcLLNal (Fig. 3), but Mdm2 was degraded 18 h after lactacystin treatment and 24 h after AcLLNal treatment, indicating that the accumulated p53 is biologically active, based on transactivation of the p53 target genes encoding $p21^{Waf1}$ and Mdm2 [32–35]. A similar accumulation of wild-type p53 has been reported in apoptosis of MOLT-4 cells induced by the proteasome inhibitor LLLal [4,12] and in

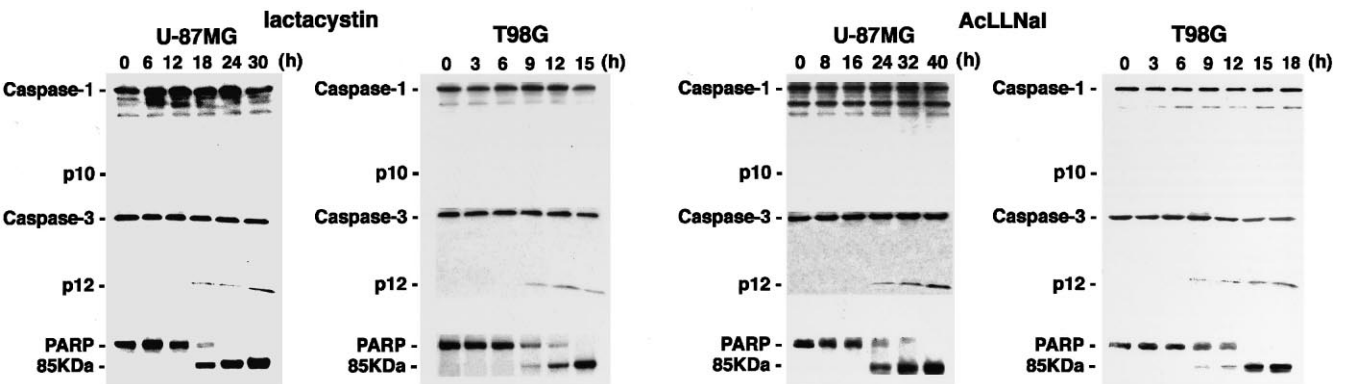

Fig. 4. Immunoblot analysis showing no activation of caspase-1 but activation of caspase-3 and cleavage of PARP in human glioma cells (U-87MG and T98G). Cells were exposed to 50 μM lactacystin for 30 h in U-87MG and for 15 h in T98G, or to 50 μM AcLLNal for 40 h in U-87MG and for 18 h in T98G. Formation of an active 10 kDa caspase-1 fragment was not detected during exposure to lactacystin or AcLLNal, suggesting no activation of caspase-1, whereas an active 12 kDa caspase-3 fragment was formed in both U-87MG and T98G cells, indicating activation of caspase-3. Cleavage of PARP into the specific 85 kDa apoptotic fragment was concomitant with the activation of caspase-3. Similar results were achieved in three separate experiments.

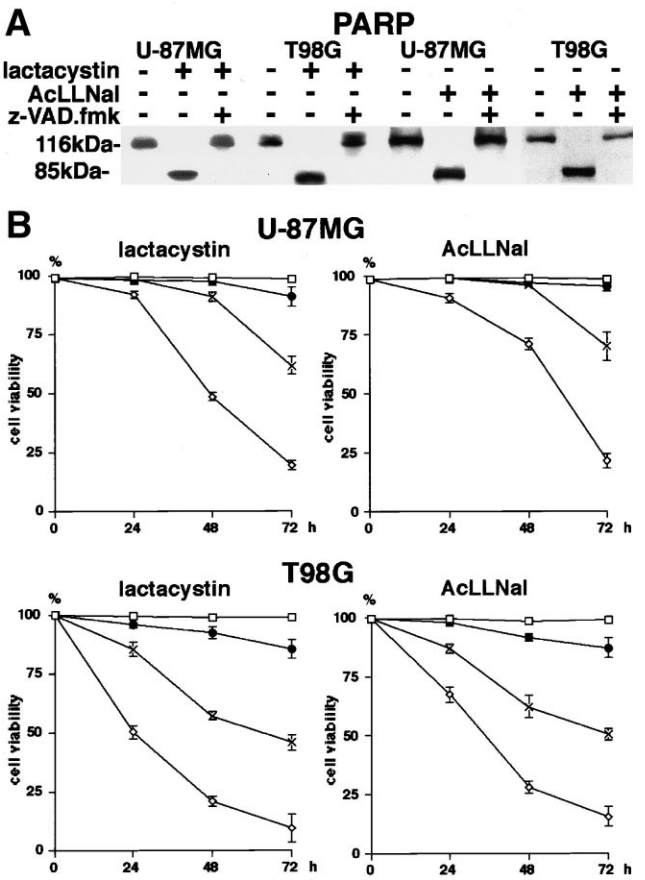

Fig. 5. Effects of z-VAD.fmk and Ac-DEVD.fmk on cell viability. A: z-VAD.fmk (100 μM) or Ac-DEVD.fmk (100 μM) was added to the culture medium 60 min prior to addition of 50 μM lactacystin or 50 μM AcLLNal and thereafter every 24 h. After treatment with either proteasome inhibitor for the indicated times, U-87MG and T98G cells were subjected to immunoblotting of PARP and examination of cell viability. In a representative immunoblot of PARP, the induction of the 85 kDa PARP apoptotic fragment by lactacystin treatment for 30 h in U-87MG (lane 2) and for 15 h in T98G (lane 5) or by AcLLNal treatment for 40 h in U-87MG (lane 8) and for 18 h in T98G (lane 11) is blocked in the presence of z-VAD.fmk in U-87MG (lanes 3 and 9) and in T98G (lanes 6 and 12). B: Cell viability graphs: open squares, control; open diamond, treatment with lactacystin or AcLLNal; closed circles, treatment with lactacystin or AcLLNal in the presence of z-VAD.fmk; crosses, treatment with lactacystin or AcLLNal in the presence of Ac-DEVD.fmk; vertical lines, standard deviations. The data shown were obtained from three separate experiments.

apoptosis of Rat-1 and PC12 cells induced by the proteasome inhibitors PSI and MG115 [14]. These results are consistent with evidence that the proteasome is involved in the ubiquitin-dependent proteolytic pathway through which wild-type p53 is degraded [2–4]. Wild-type p53 played a key role in PSI- or MG115-induced apoptosis, because the apoptosis was inhibited by expression of dominant-negative p53 [14]. However, the present lactacystin- or AcLLNal-mediated apoptosis occurs even in mutant p53-positive T98G cells, suggesting that proteasome inhibitor-induced apoptosis is rather p53-independent. Immunoreactive levels of mutant p53 in T98G cells remained unchanged by treatment with lactacystin or AcLLNal, but immunoreactive Mdm2 in T98G cells was degraded in a later stage of the treatment (Fig. 3) and immunoreactive $p21^{Waf1}$ in T98G cells became detectable as early as 3 h after the treatment (Fig. 3). The later degradation of Mdm2 by lactacystin or AcLLNal treatment in U-87MG and T98G cells may be due to proteolysis by activated caspase-3 [36]. Up-regulation of $p21^{Waf1}$ has been reported recently in the human breast cancer cell lines MCF-7 and MDA-MB-468 undergoing apoptosis induced by 10-hydroxycamptothecin and camptothecin through p53-dependent and -independent pathways [37]. In addition to $p21^{Waf1}$, another inhibitor of cyclin-dependent kinase, $p27^{Kip1}$, was also up-regulated independently of p53 protein status at least 6 h after lactacystin treatment or 8 h after AcLLNal treatment (Fig. 3), consistent with previous reports that the proteasome is involved in the ubiquitin-dependent proteolysis of $p27^{Kip1}$ [6,7,13].

To examine whether the up-regulation of short-lived proteins in response to the inhibition of the proteasome is implicated in apoptosis, glioma cell lines were pretreated for 2 h with 5 μg/ml cycloheximide and then grown in the presence of proteasome inhibitor and 1 μg/ml cycloheximide. The pretreatment with cycloheximide increased the cell viability from 71.1 ± 2.6% to 95.6 ± 1.2% at 48 h after AcLLNal treatment in U-87MG and from 67.6 ± 3.3% to 97.4 ± 1.0% at 24 h after AcLLNal treatment in T98G cells, suggesting that the accumulation of short-lived proteins is associated with proteasome inhibitor-mediated apoptosis. The accumulation of short-lived proteins such as p53 and $p27^{Kip1}$ in response to the inhibition of ubiquitin-dependent degradation has been reported to cause caspase-3 and -6-independent apoptosis [38]. Recently, the overexpression of $p27^{Kip1}$ alone has been reported to trigger apoptosis in several different human cancer cell lines as well as Rat-1 and IMR90 (human lung fibroblasts) cells [39,40]. Results from $p21^{Waf1}$ knockout animals and other experiments show unequivocally that $p21^{Waf1}$ is not required for apoptosis [41,42].

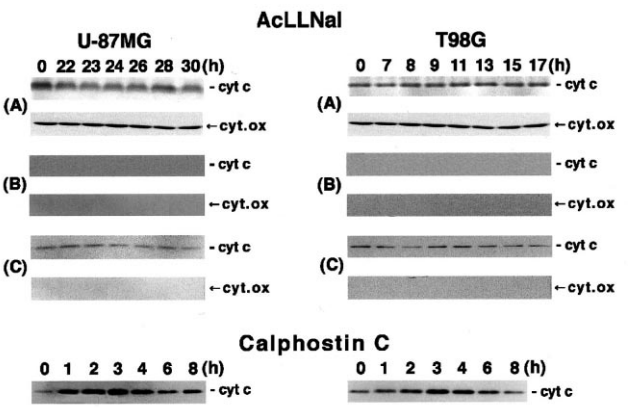

Fig. 6. Immunoblot analysis of cyt *c* and cytochrome oxidase (cyt ox) in mitochondrial (A) and S100 (B) or S10 (C) cytosolic fractions of U-87MG and T98G cells treated with 50 μM AcLLNal for the time indicated. Immunoreactive cyt *c* and cyt ox are detected in mitochondrial fractions of both cells and remain unchanged in levels during AcLLNal treatment. Immunoreactive cyt ox was not detected in S10 and S100 cytosolic fractions of both cells during AcLLNal treatment, while immunoreactive cyt *c* was detected in S10 cytosolic fractions but not in S100 cytosolic fractions of both cells and remained unchanged in level during AcLLNal treatment. Similar results were achieved in three independent experiments of AcLLNal- and lactacystin-induced apoptosis. Immunoreactive cyt *c* in S10 cytosolic fractions of both cells is increased in calphostin C-induced apoptosis, as shown in the lowermost immunoblot.

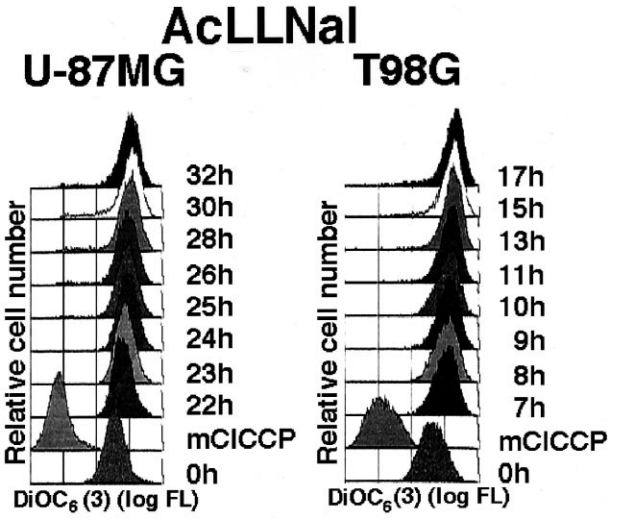

Fig. 7. U-87MG and T98G cells were treated with 50 μM AcLLNal for the indicated times and incubated with $DiOC_6(3)$, followed by FACS analysis. Data for $DiOC_6(3)$ represent log fluorescence versus relative cell number. As a control for specificity of $DiOC_6(3)$ labeling, an aliquot of the control untreated cells was exposed to the protonophore mCICCP for 15 min prior to incubation with $DiOC_6(3)$. Similar results were obtained in three separate experiments of AcLLNal- and lactacystin-induced apoptosis. FL, fluorescence.

3.3. Inhibition of proteasomal activity induces activation of caspase-3-like proteases

Immunoblot analysis of caspase-1 showed no formation of active 10 kDa caspase-1 fragment in both U-87MG and T98G cells during treatment with lactacystin or AcLLNal (Fig. 4), suggestive of no activation of caspase-1. In contrast, immunoreactive 32 kDa procaspase-3 in both U-87MG and T98G cells was degraded into an active 12 kDa caspase-3 fragment by proteolytic processing as early as 18 h in U-87MG cells and 9 h in T98G cells after treatment with lactacystin or as early as 24 h in U-87MG cells and 9 h in T98G cells after treatment with AcLLNal (Fig. 4), and the total amounts of 32 kDa procaspase-3 and 12 kDa caspase-3 fragment remained unchanged during treatment with either proteasome inhibitor, suggesting activation of caspase-3 [43–46]. In addition, a C-terminal 85 kDa PARP apoptotic fragment was generated concomitantly with the formation of the active 12 kDa caspase-3 fragment in both U-87MG and T98G cells (Fig. 4), supporting activation of caspase-3-like proteases [47–49]. Two tetrapeptide derivatives, Ac-YVAD.cmk and Ac-DEVD.fmk, which are relatively selective inhibitors of caspase-1 and -3, respectively [45], were used to preferentially block one or the other group of cysteine proteases. Ac-YVAD.cmk (100 μM), Ac-DEVD.fmk (100 μM), or z-VAD.fmk (100 μM; broad-spectrum caspase inhibitor) was added to the culture medium 60 min prior to the treatment with lactacystin or AcLLNal and thereafter every 24 h. A marked inhibition of caspase-3-like proteases in the presence of z-VAD.fmk was confirmed by the absence of the C-terminal 85 kDa PARP fragment induced by lactacystin or AcLLNal, as shown in Fig. 5, and the induction of oligonucleosomal DNA laddering by lactacystin could be completely blocked by treatment with Ac-DEVD.fmk or z-VAD.fmk in both U-87MG and T98G cells but not by treatment with Ac-YVAD.cmk (Fig. 2), confirming again the activation of caspase-3- but not caspase-1-like proteases. The induction of cell death was delayed more effectively in the presence of z-VAD.fmk than in the presence of Ac-DEVD.fmk, as shown in Fig. 5, suggesting that caspases other than caspase-3 are involved in the proteasome inhibitor-mediated apoptosis.

3.4. Inhibition of proteasomal activity induces no cyt c release or mitochondrial dysfunction

There was no evidence of significant changes in immunoreactive levels of Bcl-2, Bcl-x_L, Bax, Bad, and Bak in lactacystin- or AcLLNal-induced apoptosis of both U-87MG and T98G cells (Fig. 3). Bcl-2 and Bcl-x_L, located on the outer mitochondrial membrane, are reported to inhibit cyt *c* release, thereby blocking the apoptotic process [50–52]. Therefore, we examined by immunoblot analysis whether cyt *c* is released from mitochondria into cytosol in lactacystin- or AcLLNal-induced apoptosis. Immunoreactive cyt *c* was detected in S10 cytosolic fractions but not in S100 cytosolic fractions of U-87MG and T98G cells during AcLLNal treatment, and the immunoreactive levels of cyt *c* in S10 cytosolic and mitochondrial fractions were not changed before and after the activation of caspase-3 (Fig. 6), suggesting no release of cyt *c*. The presence of cytochrome oxidase in the mitochondrial fraction and its absence in the S10 and S100 cytosolic fractions seem to verify the adequacy of the present subcellular fractionation. In addition, cyt *c* release into cytosol was shown by immunoblot analysis of S10 and S100 cytosolic fractions and immunohistochemistry in calphostin C-induced apoptosis of the same glioma cell lines (Fig. 6 and unpublished data, respectively).

$DiOC_6(3)$-based measurement of inner mitochondrial membrane potential demonstrated no dissipation of $\Delta\Psi_m$ before the activation of caspase-3, as shown in AcLLNal-induced apoptosis (Fig. 7). Acute exposure of cells to the protonophore mCICCP confirmed that the $DiOC_6(3)$ staining was dependent on the inner mitochondrial membrane potential, thus verifying the specificity of this assay. Thus, the absence of $\Delta\Psi_m$ dissipation in the present proteasome inhibitor-induced apoptosis is associated with the absence of both cyt *c* release into cytosol and changes of Bcl-2-related proteins. Downstream caspases can become activated generally via at least two pathways: one dependent on mitochondria and another on the upstream caspases, such as those activated by Fas and some other TNF family receptors. The cytoplasmic domains of some TNF receptors, such as Fas/APO-1/CD95, are linked to caspases via FADD/MORT-1 adaptor protein [53,54]. After binding of Fas-ligand, caspase-8 and possibly caspase-10 are recruited to the cytoplasmic domain of the Fas receptor and become activated by proteolysis [25,26]. Once activated in cells, these upstream caspases can then cleave and activate certain downstream caspases, such as caspase-3, which are directly responsible for the proteolytic cleavage of cell death substrate proteins and the actual apoptotic demise of the cell [55–57]. It is important to recognize, however, that cross-talk between these two pathways is extensive. For example, upstream and downstream caspases, once activated, can act on yet unknown substrates in the mitochondrial membrane, thereby inducing the dissipation of $\Delta\Psi_m$ and the release of apoptogenic proteins [58–60]. However, in the present proteasome inhibitor-induced apoptosis, neither cyt *c* release nor $\Delta\Psi_m$ loss is observed not only before but also after

the activation of caspase-3 (Figs. 6 and 7). Thus, the proteasome inhibitor induces mitochondria-independent apoptosis.

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