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The mechanism underlying apoptosis induced by proteasome inhibition in leukemic Jurkat and Namalwa cells was investigated in this study. The proteasome inhibitor lactacystin differentially regulated the protein levels of proapoptotic Bcl-2 family members and Btk was accumulated at the mitochondria. Btk overexpression sufficed to induce apoptosis in these cells. Detailed examination along the respiration chain showed that lactacystin compromised a step after complex III, and exogenous cytochrome c could overcome this compromise. Probably as a result, the succinate-stimulated generation of mitochondrial membrane potential was significantly diminished. Bcl-xL, interacting with Btk in the cells, and Bcl-xL overexpression prevented cytochrome c leakage out of the mitochondria, corrected the mitochondrial membrane potential defect, and protected the cells from apoptosis. These results show that proteasomes can modulate apoptosis of lymphocytes by affecting the half-life of Bcl-2 family members, Btk being one of them. The Journal of Immunology, 2001, 166: 3130–3142.

Proteasomes, large protease complexes in cells, are located in the cytoplasm and nucleus (1). They have at least five distinct peptidase activities, i.e., chymotrypsin-like, tryptic-like, peptidylglutamyl peptide-hydrolyzing, branched chain amino-acid-prefering, and small neutral amino acid-prefering activities (2). The first three activities have been well-characterized. Proteasomes are the major intracellular machinery for protein degradation (3) and were regarded as housekeeping enzymes disposing spent proteins. However, it has become increasingly clear that they play critical and active roles in regulating many different cellular functions. These are achieved by their ability to timely, selectively, and irreversibly destroy regulatory protein factors and to process precursors of regulatory factors into active ones. For example, the degradation of several important regulators of cell activation and proliferation, such as cyclin 2, cyclin 3, cyclin B, p53, p27kip1, IκBα, and c-Jun proteins, occurs via the proteasome pathway (4–11); transactivating NF-κB matures after cotranslational processing of its precursor peptide by proteasomes (12). Selective ubiquitination of proteins is an important mechanism controlling the discriminative nature of protein degradation via proteasomes (1).

Apoptosis is an essential cellular event in organ development and tissue remodeling. In the immune system, apoptosis is required for positive and negative selection of T and B cells, and for maintaining homeostasis of peripheral lymphocytes. It is also a mechanism for lymphocytes to kill their target cells. Abnormal apoptosis could result in pathological conditions such as autoimmune diseases and failure to terminate infectious diseases, whereas properly induced apoptosis might be useful in controlling undesirable immune responses. Because proteasomes degrade 70–90% of cellular proteins (3), it is conceivable that many cellular events, including apoptosis, are directly or indirectly controlled by them. A specific proteasome inhibitor, lactacystin (LAC),6 can repress three major peptidase activities of proteasomes (i.e., chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide-hydrolyzing activities), but does not affect other proteases, such as calpain, cathepsin B, chymotrypsin, trypsin, and papain (13). By using this inhibitor, we have demonstrated previously that proteasome inhibition results in apoptosis of cycling T cells (14).
In this study, we further investigated the underlying mechanisms in Jurkat T cells (JC) and Namalwa B cells (NC). Our results suggest that proteasomes play a critical role in maintaining the balance between pro- and anti-apoptotic factors and in maintaining proper functions of the mitochondria (Mito). These are critical events that decide the fate of cells.

**Materials and Methods**

**Reagents**

RPMI 1640, FCS, penicillin-streptomycin, and t-glutamine were purchased from Life Technologies (Burlington, Ontario, Canada). LAC was obtained from Dr. E. J. Corey (Ref. 13; Harvard University, Boston, MA). The caspase inhibitor benzoxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (zVAD.fmk) was purchased from Enzyme Systems Products (Livermore, CA). The fluorogenic caspase substrate Ac-Asp-Glu-Val-Asp-aminomethylcoumarin was purchased from Bachem Bioscience (King of Prussia, PA). Mouse mAb (clone 7H12C12) against cytochrome c (CytC) was obtained from BD PharMingen (San Diego, CA). Rabbit Abs against Bax, Bak, and Bad, normal rabbit IgG, and goat Abs against Bik were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Swine anti-goat Ab was obtained from Callau (San Francisco, CA). Mouse mAb against hemagglutinin (HA) and rabbit Ab against poly(ADP-ribose) polymerase (PARP) were purchased from Roche Diagnostics (Laval, Quebec). Digitonin (Dig), succinate (Suc), antimycin A (Anti A), tetramethyl-p-phenylenediamine (TMPD), ascorbate (Asc), carbonyl cyanide m-chloro-phenylhydrazine (CCCP), carbonyl p-trifluoroethoxyphenylhydrazine (FCCP), and camptothecin (Campt) were obtained from Sigma (Oakville, Ontario, Canada). 5,5'-Tetrachloro-1,1'-tetraethyl-benzimidazole-carboxylate (JC-1) was purchased from Molecular Probes (Eugene, OR). ECL kits and protein G-agarose were obtained from Amersham (Oakville, Ontario, Canada). [35S]labeled (1,175 Ci/mmol), and Biotrans nylon membranes were obtained from ICN Pharmaceuticals (Mississauga, Ontario, Canada).

**Cell culture**

JC and NC were cultured in RPMI 1640 supplemented with 10% FCS, t-glutamine, and antibiotics as described elsewhere (15).

**Electron microscopy**

JC were examined by electron microscopy as described by Tsao and Du-guid (16).

**DNA laddering assay**

The assay was performed according to a protocol by Liu et al. (17) with some modifications (14).

**The DNA filter elution assay**

JC or NC were labeled with [3H]thymidine (0.02 µCi/ml) for 24 h and chased in isotope-free medium overnight before drug treatment. After drug treatment, the samples were processed as described earlier (18). DNA fragmentation was determined by liquid scintillation as the fraction of fragmented DNA relative to total DNA. Background DNA fragmentation (in untreated cells) was deducted in the final results presented according to the formula: \( (F_0 - F_1)/(1 - F_0) \times 100 \), where \( F \) and \( F_0 \) represent DNA fragmentation in treated and untreated cells, respectively.

**Caspase 3-like activity (DEVDase) assay**

JC were washed twice with ice-cold PBS and lysed at 4°C at a density of \( 1.0 \times 10^7 \) cells/ml in lysis buffer containing 10 mM HEPES, 20 mM NaCl, 80 mM KCl, 5 mM MgCl2, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 0.15 U/ml aprotinin, 10% glycerol, and 0.1% Nonidet P-40, pH 7.4. After incubation at 4°C for 10 min with gentle agitation, the samples were centrifuged at 4°C at 10,000 \( g \) for 10 min to remove cell debris. The supernatants were used as sources of mitochondrial fractions, and the cytoskeletons were centrifuged at 100,000 \( g \) for 15 min to remove unbroken cells and cellular debris. The supernatants were further centrifuged at 10,000 \( g \) for 30 min. The pellets from these centrifugations represented mitochondrial fractions, and the supernatants were used as cytosolic fractions. The mitochondrial but not the cytosolic fraction thus prepared could resolve (data not shown). In some experiments, the mitochondrial fractions were lysed by a buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 mM NaCl, and 1% Nonidet P-40 and centrifuged at 10,000 \( g \) for 15 min to pellet the debris. The supernatants were used as sources of mitochondrial protein in the immunoblotting.

Mito from rat kidney proximal tubules (RKM) served as positive controls for normal respiratory functions. They were isolated by differential centrifugation after homogenization in a buffer containing 250 mM sucrose, 10 mM HEPES-Tris (pH 7.5), and 250 mM EDTA. Cell debris was removed by centrifugation at 2,500 \( g \) for 15 min, and the mitochondria were precipitated by centrifugation at 10,000 \( g \) for 30 min. They were finally washed with the same buffer in the absence of EDTA. Protein concentrations of the mitochondrial suspensions were measured after solubilizing the Mito in 0.1% SDS.

The purity of the mitochondrial and cytosolic fractions was determined by the activities of glutamate dehydrogenase and lactate dehydrogenase in each fraction. Contamination of cytosolic protein in the mitochondrial fraction was <35%, whereas contamination of mitochondrial protein in the cytosolic fraction was <15% as described previously (21).

**Immunoblotting**

Immunoblotting was used to evaluate PARP cleavage and the protein levels of Bik, Bax, Bak, Bad, Bel-2, and CytC. The general protocol can be found in our previous publication (14). For PARP, Bax, Bak, and Bad detection, the membranes were hybridized with specific rabbit Abs at dilutions suggested by the manufacturers. For CytC and HA-Bcl-xL detection, the membranes were hybridized with mouse mAb against pigeon CytC or mouse mAb against the HA tag, respectively. The signals on the membranes were detected by ECL. For the detection of Bik, goat anti-Bik Ab was used as the first Ab in immunoblotting. The membranes were then either hybridized with a swine anti-goat Ab followed by [125I]-protein A or ECL for signal detection.

**Northern blot analysis**

The method is described in our previous publication (22). Briefly, total cellular RNA of JC and NC was extracted with the guanidine/CsCl method and used in the Northern blots. A 402-bp Bik cDNA lacking the transmembrane domain was labeled by [32P] with random primers and was served as a probe. Bik cDNA has been described in our previous publication (23).

**Metabolic labeling of Bik**

JC were labeled with Tran-35S (1,175 Ci/mmol, 0.9 mCi/20 \( \times 10^7 \) cells/5 ml; ICN Pharmaceuticals) for 20 min, then cultured in normal RPMI 1640 medium with 10% normal FCS for the absence or presence of 100 \( \mu \)M of 8-bromo-cAMP for 6 h. The cells of each treatment (20 \( \times 10^6 \) cells/treatment) were lysed with 0.5 ml of lysis buffer in the presence of protease inhibitors (24) for 1 h on ice. After spinning, the supernatants (0.7 mg protein/treatment) were preclared for 1 h at 4°C with 10 \( \mu \)l of normal goat serum and 20 \( \mu \)l of protein G-agarose. The supernatants were then incubated with goat anti-Bik Ab overnight at 4°C. Immune complexes were precipitated with 50 \( \mu \)l of protein G-agarose, resolved in 4–20% gradient SDS-PAGE, and transferred to polyvinylidene difluoride. Signals were visualized by autoradiography, and band intensities were determined by densitometry.

**Immunoprecipitation**

Bcl-xL-transfected NC were lysed in 1% Brij lysis buffer containing proteinase inhibitors as described previously (24). The cleared lysate (400 \( \mu \)g protein/sample) was incubated with mouse anti-HA mAb (10 \( \mu \)g) on ice for 1 h. To recover the immune complexes formed, 5 \( \mu \)g of rabbit anti-mouse IgG and 30 \( \mu \)l of Pansorbin (formalin-fixed Staphylococcus aureus Cowan 3131The Journal of Immunology 3131

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I) were added to the lysate, and the samples were kept at 4°C with rotation for 2 h. Pansorbin was washed four times with PBS, and the immune complexes were eluted with an SDS-PAGE sample buffer followed by electrophoresis on 7.5% SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride membranes and blotted sequentially with goat anti-Bik Ab and a mAb against the HA tag.

Transient transfection

JC and NC were transfected by electroporation in serum-free RPMI 1640 medium. Ten million cells/0.8 ml medium/10 μg plasmid/cuvette were electroporated at room temperature. The electroporation settings were 270 V/960 μF for NC and 360 V/960 μF for JC.

Measurement of mitochondrial membrane potential (ΔΨm)

ΔΨm was measured with JC-1 (25). JC-1 (0.1 μM) uptake by Mito in live cells (Dig-permeabilized JC and NC) as well as by purified Mito from rat kidney, JC, and NC at 37°C was monitored continuously with a spectrofluorometer ( Photon Technology International, South Brunswick, NJ). The excitation wavelength was 490 nm (slit width, 2 nm) and the emission wavelength was 590 nm (slit width, 4 nm). The signals were recorded by Felix (Version 1.1) software (Photon Technology International, South Brunswick, NJ). The incubation buffer, substrates, and inhibitors for measurement of ΔΨm were identical with those used in the respiration assays (as described below). For each determination, 0.5 × 10⁶ cells or purified Mito equivalent to 50 μg of protein from JC and NC were used.

Respiration assay

Electron transport in Mito was assessed according to oxygen consumption during respiration. JC (30 × 10⁶ cells/ml) or RKM (0.5 mg protein/ml) were incubated in a 1-ml thermostated chamber at 37°C in respiration buffer (200 mM sucrose, 5 mM MgCl₂, 5 mM KH₂PO₄, and 30 mM HEPES-Tris, pH 7.5). Various reagents (0.005% Dig, 10 mM Suc, 1 mM Asc, 0.4 mM TMPD, 1 μM CCCP, 1 μM FCCP, 0.1 μM rotenone, 50 mM antimycin A, 1 mM KCN, and 100 μM CytC) were added during the assay. The respiration rate was measured polarographically with a Clarke oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). Oxygen concentration was calibrated with air-saturated buffer in a hypoxanthine-xanthine oxidase/catalase system (chemical zero). Oxygen consumption was registered continuously by a MacLab/8 multichannel recorder (Worx, Dover, NH) connected to a Macintosh SE computer (Apple Computer, Cupertino, CA) and was analyzed with MacLab Chart software (version 3.3.4; Worx). The oxygen consumption rates are expressed as nanograms of atoms of oxygen per minute.

Results

Blocking proteasome activity results in JC apoptosis

We have shown previously that proteasome inhibition results in the death of cycling T cells (14). The mode of cell death induced by LAC was investigated here in fast-growing JC. According to
electron microscopy (Fig. 1, A and B), there was apparent nuclear condensation in JC treated with LAC at 6 μM for 24 h. DNA laddering in the cells could be detected as early as 6 h after LAC treatment (Fig. 1C). Thus, blocking proteasome activity induces apoptosis in these cells.

Opposite observations concerning the role of the proteasome in apoptosis have been reported. Several proteasome inhibitors including LAC can inhibit thymocyte apoptosis induced by irradiation, dexamethasone, or PMA (26). LAC at low concentrations (1–10 μM) can repress apoptosis of a T cell hybridoma induced by CD3 cross-linking (27). Apoptosis of nerve growth factor-deprived sympathetic neurons is also inhibited by LAC (28). We wondered whether the difference between our results and the reported anti-apoptotic effects of proteasome inhibitors was caused by different degrees of inhibition of proteasome activity. To address this question, JC were triggered to apoptosis with PHA, and different concentrations of LAC were added to the system. No antiapoptotic effect of LAC could be observed at concentrations tested ranging from 6 to 0.07 μM, according to DNA fragmentation (Fig. 1C). Moreover, from 2 μM and up, LAC further enhanced the apoptosis induced by PHA. Because the LAC concentrations used here covered the full spectrum from complete inhibition to no inhibition of proteasome activity according to our previous study (29), we conclude that proteasome inhibition only promotes apoptosis but has no antiapoptotic effect in our model.

To assess more quantitatively the apoptosis induced by proteasome inhibition, we used a DNA filter elution assay. [14C]Thymidine-labeled JC were treated with LAC at different concentrations (from 0.75 to 10.0 μM) for 6 or 24 h, and DNA fragmentation was measured according to small DNA fragments released from the nuclei. The results showed that the DNA fragmentation of JC increased dose- and time-dependently after LAC treatment (Fig. 1D). Twenty-four-hour treatment at concentrations above 6 μM caused ~90% DNA fragmentation.

**DEVIdase is activated in apoptosis induced after proteasome inhibition**

In most known pathways of apoptosis, various caspases are implicated in the initiation or execution phases. Is this also the case in apoptosis induced by proteasome inhibition? A broad-spectrum caspase inhibitor, zVAD.fmk, could effectively inhibit LAC-induced DNA fragmentation in JC (Fig. 2A). Inhibition was dose-dependent, whereas zVAD.fmk by itself had no effect. This result shows that caspase activation is required in LAC-induced DNA fragmentation.

It is known that PARP is a substrate of caspase-3 (30), which is an execution-phase caspase (31). Therefore, PARP cleavage was tested in JC treated with LAC. A 115-kDa PARP was cleaved into 89-kDa and 24-kDa bands (indicated by arrows in Fig. 2B) after LAC treatment. The 24-kDa band was a characteristic product after DEVIdase activation (32) and appeared as early as 5 h after the blockage of proteasome activity. An enzyme assay with a fluorogenic substrate specific for DEVIdase was used to further confirm DEVIdase activation ex vivo. As shown in Fig. 2C, DEVIdase was indeed augmented in JC after 5 h of proteasome inhibition. Taken together, the above results show DEVIdase activation in these cells after proteasome inhibition.

**Proteasome inhibition results in accumulation of Bik protein but not several other pro-apoptotic members of the Bcl-2 family**

The function of proteasomes is to degrade proteins, and logically, a simple mechanism by which a proteasome inhibitor induces apoptosis could be to differentially block the degradation of proapoptotic factors and thus shift the balance between antiapoptotic and proapoptotic factors. In fact, we observed accumulation of Bik protein but not several other pro-apoptotic members of the Bcl-2 family (Fig. 3A). Thus, blocking proteasome activity induces apoptosis in these cells.

**FIGURE 2.** DEVDase is activated after proteasome inhibition. A, LAC-induced DNA fragmentation is inhibited by a broad spectrum caspase inhibitor, zVAD.fmk. JC were treated with LAC (6 μM) in the absence or presence of zVAD.fmk (0.4–33.3 μM) for 6 h. They were harvested and their DNA was analyzed by DNA laddering assay. B, PARP cleavage during LAC-induced apoptosis. JC were cultured in medium (Cont) or in the presence of 6 μM LAC for 1, 3, or 5 h as indicated. They were harvested and the cell lysates were analyzed by immunoblotting using a rabbit anti-PARP Ab. The arrows show cleaved PARP fragments (89 and 24 kDa). C, DEVIdase activity in JC after proteasome inhibition. JC were treated with LAC (6 μM) for 5 h, and DEVIdase activity in 600 μg of lysate protein of the treated cells was measured by a continuous 400-s kinetic assay by using Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (AMC) as substrate. The amount of free AMC (pmol) generated is shown, and a representative result of two similar experiments is presented.
proapoptotic factors in favor of the latter, hence apoptosis. This mechanism is applicable to members of the Bcl-2 family, some of which are antiapoptotic, and the others are proapoptotic (33). Therefore, we studied the effect of LAC on the protein levels of several proapoptotic Bcl-2 family members. As shown in Fig. 3A, Bik was present mainly in Mito and was hardly detectable in the cytosol of JC. After 4–6 h of LAC treatment, the Bik level increased 70–100% in the mitochondrial fraction (Fig. 3, A and B), and became detectable in the cytosol (Fig. 3A). Several other pro-apoptotic members such as Bax, Bak, and Bad were also present in the mitochondrial fraction, but their levels remained unchanged in this fraction (Fig. 3B) as well as in the cytosolic fraction (data not shown) after LAC treatment.

To ascertain that Bik accumulation after proteasome inhibition was not an event unique to JC, we examined the Bik level in a human B cell line, NC. A 6-h LAC treatment of NC resulted in Bik increase as with JC (Fig. 4, A and B). Like LAC, a protein kinase inhibitor, staurosporine (STS), could also induce apoptosis in JC and NC (data not shown), but it did not augment Bik levels (STS). The membranes in Figs. 3 and 4 were probed more than once with different Abs to rule out artifacts caused by uneven protein loading. Even protein loading was also assured by bands recognized by anti-cytochrome oxygengase IV appearing in the immunoblots (Fig. 4, bottom).

With Northern blot analysis, we have shown that there was no increase in Bik mRNA in JC, Bcl-xL-transfected NC, or wild-type NC after 6 h of LAC treatment (Fig. 5A). This result suggests that the accumulation of Bik protein after LAC treatment is unlikely attributable to enhanced protein synthesis. We further confirmed with metabolic labeling that LAC inhibited Bik protein turnover (Figs. 5B), and this was quantified by densitometry (Fig. 5C). Thus, the accumulation of Bik was due to its reduced degradation.

Bcl-xL overexpression protects NC from apoptosis induced by proteasome inhibition

One way to test our hypothesis that the imbalance between Bik and antiapoptotic Bcl-2 family members results in apoptosis was to ascertain whether overexpression of antiapoptotic factors would

FIGURE 3. Proapoptosis inhibition differentially increases levels of Bik in JC. Proteins from Mito and cytosol of JC with or without LAC treatment (6 μM) were used for immunoblotting as indicated. A, Immunoblot of Bik in Mito and cytosol. JC were treated with LAC for 5 h. B, Immunoblot of Bik, Bax, Bak, and Bad in Mito of JC. JC were treated with LAC for 4 or 6 h.

FIGURE 4. Proapoptosis inhibition modulates Bik levels in both JC and NC. Mito from JC (A) and NC (B) were treated with STS (0.3 μM 6 h) or LAC (6 μM, 6 h). Bands recognized by anti-cytochrome oxygengase IV were shown in the bottom panels to indicating even protein loading.
overcome the effect of LAC and tip the balance in favor of cell survival. Thus, NC were stably transfected with pCEP4-HA-Bcl-xL to express Bcl-xL fused with a HA tag sequence (19). We then tested whether Bcl-xL-transfected NC were resistant to apoptosis induced by LAC. Wild-type NC underwent DNA fragmentation when treated with a DNA topoisomerase I inhibitor Campt (1 μM) or LAC (1.5 μM) for 24 h, according to a DNA laddering assay (Fig. 6A), and the degree of laddering was reduced in Bcl-xL-transfected cells (Fig. 6B). The protective role of Bcl-xL in this model was assayed quantitatively by DNA filter elution assay. Wild-type and Bcl-xL-transfected NC were treated with LAC of different concentrations (0.75–10 μM) for 0–96 h. Under all the conditions tested, Bcl-xL-transfected cells had significantly reduced DNA fragmentation (Fig. 6C). This indicates that Bcl-xL overexpression protects these cells from apoptosis induced by proteasome inhibition, although the protection is not complete.

A likely mechanism for the protective effect of Bcl-xL is that it neutralizes the pro-apoptotic effect of Bik. If so, Bcl-xL might physically interact with Bik. We tested this possibility in NC overexpressing exogenous Bcl-xL, because due to the low level of endogenous Bcl-xL in wild-type lymphocytes, the putative interaction between Bik and Bcl-xL is difficult to detect. As shown in Fig. 6D, the immune complexes precipitated by anti-HA, which binds tags in overexpressed exogenous Bcl-xL, contained Bik signals. The same membrane when hybridized with anti-HA mAb revealed Bcl-xL signals, indicating that exogenous Bcl-xL was indeed expressed in the transfected NC. This result suggests that Bik and Bcl-xL interact with each other in these cells.

Expression of exogenous Bik leads to apoptosis of lymphocytes

Does augmentation of the Bik level, as seen after proteasome inhibition, suffice for the induction of apoptosis in lymphocytes? To answer this question, we transiently transfected NC and JC with a Bik expression construct pCDNA3-HA-Bik, which has been described in a previous publication (23), and apoptosis of these cells underwent DNA filter elution assay at 24, 48, and 72 h after transfection. As shown in Fig. 7, electroporation caused 15–20% and 45% DNA fragmentation, respectively, in NC and JC transfected with empty vectors (blank columns). Above such a background, the Bik construct-transfected NC and JC (dotted columns, first and last panels) had significantly augmented DNA fragmentation, which was more pronounced and occurred earlier in JC. This indicates that augmentation of Bik expression alone is indeed sufficient to cause apoptosis in lymphocytes. An additional experiment showed that exogenous Bik expression in Bcl-xL-protected cells did not lead to augmented apoptosis (Fig. 7, middle), indicating that the apoptosis induced by pCDNA3-HA-Bik transfection was not attributable to nonspecific cytotoxicity of the plasmid.
Collapse of Δψₘ after proteasome inhibition and its prevention by Bcl-x₁

Because Bik is mainly located on the outer membrane of Mito, these organelles are then implicated in apoptosis induction. This notion is supported by observations from other studies (reviewed in Ref. 34). Cells undergoing apoptosis show an early reduction of Δψₘ. Permanent collapse of Δψₘ marks a point-of-no-return during apoptosis induction in most cases, although it is not known whether Δψₘ collapse per se is responsible for triggering apoptosis.

To evaluate the involvement of Mito in LAC-induced apoptosis, we used JC-1 fluorometric assay to measure Δψₘ driven by Suc. In the presence of Suc as a substrate, the generation of Δψₘ is maximal and depends only on electron transport chain function and permeability of the inner mitochondrial membrane, and the function of the electron transport chain is not limited by the availability of and/or access to endogenous substrates in permeabilized cells. Under such an extreme condition, potential defects were easy to be identified. Moreover, this experimental condition was identical to the one used to illustrate the effect of Bcl-x₁ overexpression in NC.
with those of the respiration studies, and the results of $\Delta \psi_m$ and respiration could thus be compared.

In untreated JC permeabilized by Dig, the addition of Suc led to JC-1 accumulation, which reached the maximum at 400 s (Fig. 8A, control (Cont)). JC-1 accumulation under this condition was due to JC-1 accumulation, which reached the maximum at 400 s (Fig. 8A). Respiration could thus be compared. Due to the reduced diffusion time of purified Mito, with respiration of Mito from untreated JC (Fig. 9D), the addition of normal RKM in this experiment resumed oxygen consumption, indicating that the assay system was functional. These results suggest defective function of the mitochondrial electron transport chain somewhere between complex II and IV.

To determine more precisely the location of the defect, the function of cytochrome oxidase (complex IV) alone was tested in respiration experiments. The respiration of purified RKM (untransfected) could be blocked at complex III by Anti A, and electron flow through complex IV could be resumed with the artificial electron donors Asc and TMPD (Fig. 9C). The addition of CCCP further accelerated electron flow (Fig. 9C). The respiration of Mito from Dig-permeabilized JC with or without LAC treatment for 2 h (Fig. 9C, curves LAC and Cont) functioned as normal RKM. Respiration was accelerated by Asc plus TMPD, further boosted by CCCP, and completely blocked by KCN (inhibitor of complex IV). However, treatment of JC with LAC for 4 h (Fig. 9D, LAC curve) led to a significant decrease of electron flow through complex IV, and this flow failed to be stimulated by CCCP, when compared with respiration of Mito from untreated JC (Fig. 9D, Cont curve). The addition of purified normal RKM in this experiment resumed oxygen consumption (Fig. 9D, LAC curve). These results show that after 4 h of proteasome inhibition, there is a defect between complex III and IV.

CytC is a low-m.w. soluble protein localized in mitochondrial intermembrane spaces and functions as an electron transport protein shuttling between complex III and IV. In most models of apoptosis, CytC leaks out of Mito in the early stage of apoptosis (35, 36). Therefore, we tested whether exogenous CytC could correct the defect in the respiration chain in LAC-treated JC. As shown in Fig. 9E, the addition of exogenous CytC had no effect on Asc-TMPD-driven and FCCP-accelerated respiration of LAC-treated RKM (LAC curve), or Dig-permeabilized untreated JC (Cont curve). However, the exogenous CytC resulted in acceleration of respiration with restoration of electron flow in JC treated with LAC for 4 h.

The results of this section show that after proteasome inhibition, the electron transport chain is compromised after complex III, and the defect likely involves CytC, which either leaks out or is functionally defective.

Bcl-xL overexpression prevents CytC leakage out of Mito after proteasome inhibition

The function of the electron transport chain was studied by respiration assay (Fig. 9) with Dig-permeabilized JC under conditions similar to those of the membrane potential assay (Fig. 8A). In these experiments, purified RKM were used as a control. Respiration of RKM on endogenous substrates was blocked by rotenone (inhibitor of NADH-dehydrogenase or complex I), resuspended by Suc (substrate of Suc-dehydrogenase or complex II), and reached its maximum after dissipation of the $\Delta \psi_m$ by CCCP (Fig. 9A). Respiration of Mito in Dig-permeabilized JC with or without LAC treatment for 2 h was similar to that of RKM (Fig. 9A). However, the treatment of JC with LAC for 4 h (LAC, Fig. 9B) significantly diminished Suc-driven respiration and prevented additional respiration stimulated by CCCP, when compared with the respiration of untreated JC (Cont). The addition of purified normal RKM into this experiment resumed oxygen consumption, indicating that the assay system was functional. These results suggest defective function of the mitochondrial electron transport chain somewhere between complex II and IV.

In a separate experiment, we found that the caspase inhibitor zVAD.fmkk could not prevent the LAC-induced compromise of $\Delta \psi_m$ (data not shown). This indicates that this mitochondrial event is upstream of caspase activation.

The electron transport chain is defective after complex III following proteasome inhibition, and the defect can be reversed by exogenous CytC

Generation of $\Delta \psi_m$ primarily depends on efficient function of the electron transport chain and the integrity of the inner mitochondrial membrane. Thus, we next asked whether there is a functional defect in the electron transport chain in Mito of LAC-treated JC.
also was decreased compared with that of untreated cells. A nonspecific band of 75 kDa recognized by anti-CytC mAb had similar intensity in all the samples, and this band served as internal controls for protein loading in immunoblotting.

We further demonstrated in NC that concomitant to the decrease of CytC levels in Mito, there was an increase of CytC in the cytosol (Fig. 10C) in LAC-treated cells (6 h), while before the treatment (0 h), CytC was not detectable in the cytosol. This indicates

FIGURE 8. Generation of Suc-driven Δψm in Mito of JC, NC, and Bcl-xL-transfected NC after proteasome inhibition. A, Δψm measured with permeabilized JC. Half a million JC were added to the respiration buffer and permeated with 0.005% Dig, Suc (10 mM), Anti A (0.5 μM), and FCCP (5 μM) were added sequentially as indicated by arrows. JC-1 fluorescence intensity of the cells was monitored for a time course up to 1400 s, and intensity is expressed as counts per second (CPS). Δψm of untreated JC (Cont) and of cells treated with 6 μM LAC for 2, 6, or 8 h was analyzed. B, Δψm measured with Mito purified from JC. Mito (50 μg of protein) purified from JC were used for the measurement of Δψm by JC-1 assay. The assay is the same as described above, except that no Dig was used and Anti A (0.1 μM) and FCCP (5 μM) were added. Mito of untreated JC (Cont), of JC treated with 6 μM LAC for 6 h, and of JC treated with 0.3 μM STS for 6 h were analyzed. C and D, Δψm measured with Mito purified from wild-type NC (C) or Bcl-xL-transfected NC (D). The assay condition was identical with that described in Fig. 6A. Mito of cells cultured in medium (Cont), of cells treated with 0.3 μM STS for 6 h, and of cells treated with 6 μM LAC for 6 h were analyzed.
that proteasome inhibition triggers the leakage of CytC from the Mito to the cytosol.

Could Bcl-xL prevent CytC leakage after proteasome inhibition? Bcl-xL overexpression in the Mito and cytosol of Bcl-xL-transfected NC was evident according to 32.7-kDa bands recognized by a mAb against the HA tag of the fusion protein (Fig. 10D), whereas in wild-type NC, it was not detectable, as expected (Fig. 10C). In Bcl-xL-transfected cells, there was no decrease of CytC in Mito, nor did CytC become detectable in the cytosol after 6 h of LAC treatment. These results show that Bcl-xL prevents CytC from leaking out of Mito.

Discussion

There are several major new findings in this study. First, we have demonstrated that a proteasome inhibitor differentially up-regulates a proapoptotic Bcl-2 member, Bik, by decreasing its degradation, and Bik up-regulation suffices to induce apoptosis in leukemic cells. Second, detailed analysis of the effect of proteasomes on the electron transport chain reveals that the proteasome activity is required for proper functioning of the chain and generation of Δψm. Third, we have extended current knowledge on the protective role of Bcl-xL to LAC-induced apoptosis and diminished Δψm and showed that this is likely achieved by direct interaction between Bik and Bcl-xL.

Bik was found to be accumulated in Mito and cytosol after proteasome inhibition, whereas several other members, such as Bax, Bak, and Bad, were not affected. Bik/NBK/Blk, BID, Hrk, NIP3, BimL/BOD, and Bad belong to a so-called “BH3-only” proapoptotic Bcl-2 subgroup (37). They are more potent in their apoptosis-inducing activity than other members that contain additional BH1.
and BH2 domains (38). BID is able to cause disruption of the outer mitochondrial membrane, because recombinant BID, especially truncated BID added to purified Mito, could lead to CytC release (38). In a cell-free Xenopus oocyte system, the BH3 domain alone can induce CytC release and activation of caspases (39). In keeping with these features of BH-3-only molecules, Bik overexpression results in decrease of $Dc_m$ (40), and recombinant Bik induces CytC release from purified Mito (41). Moreover, expression of exogenous Bik suffices to induce apoptosis as demonstrated in leukemic cells in our study and in other types of cells (23, 42, 43). Based on these results, we believe that Bik accumulation after proteasome inhibition plays an important role in apoptosis induction in our model.

Our observation that proteasome activity is required to prevent apoptosis in leukemic cells is supported by another study in which Imajoh-Ohmi et al. reported that LAC induces apoptosis in U937 cells (44). However, blocking proteasomes seems to prevent apoptosis under certain circumstances, such as dexamethasone-treated thymocytes (26) and neurons deprived of nerve growth factor (28). Is this due to different degrees of inhibition? We have covered the full spectrum of LAC concentrations and no antiapoptotic effect was observed in our model. The function of the proteasome is the same in all these cells, i.e., to degrade proteins. Why then do different cells respond to proteasome inhibitors differently? It is to be noted that the protein levels of not only proapoptotic but also antiapoptotic factors could be proteasome dependent. We speculate that under normal circumstances proteasomes maintain a dynamic equilibrium between antiapoptotic and proapoptotic factors. If this is correct, we could think of at least two scenarios in which proteasome inhibitors can cause opposite effects in apoptosis. First, it

**FIGURE 10.** CytC leaks out of Mito in LAC-treated cells, and the leakage is prevented by Bcl-xL overexpression. A, Decreased levels of CytC in Mito from LAC-treated JC and NC. JC (A) and NC (B) were cultured in medium (Cont) or treated with 0.3 μM STS or 6 μM LAC for 6 h. Their Mito were purified, and 15 μg of mitochondrial protein was loaded in each lane. The CytC band is indicated by arrows CytC, and a 75-kDa nonspecific band recognized by anti-CytC mAb reflects the even protein loading among lanes. C and D, Bcl-xL overexpression prevents mitochondrial CytC from leaking into cytosol in LAC-treated NC. Wild-type NC (C) and Bcl-xL-transfected NC (D) were either untreated (0 h) or treated with 6 μM LAC for 6 h as indicated. Mitochondrial or cytosolic proteins (40 μg/lane) from these cells were analyzed with 10% SDS-PAGE for their CytC levels by immunoblotting (upper row). The same membranes were also blotted with anti-HA mAb which recognizes exogenous but not endogenous Bcl-xL in the transfected NC (bottom row).
is possible that different types of cells or cells at different differentiation stages are using different antiapoptotic or proapoptotic factors (45–47). For example, it has been reported that in thymocytes, degradation of inhibitor of apoptosis is necessary for glucocorticoid-induced apoptosis, and the degradation is a proteasome-mediated process (48). If inhibitor of apoptosis has a significant weight in the equilibrium, this can then explain quite well why in thymocytes, proteasome inhibitors prevent but do not induce apoptosis. Second, even the same factor might have different rates of synthesis and proteasome-dependent degradation in different cells. We have shown that the levels of proapoptotic factors Bak and Bax are not changed in Jc and NC after proteasome inhibition, whereas in a pancreatic tumor cell line BxPC-3 both Bak and Bax are accumulated after LAC treatment, as reported in our recent publication (49). This suggests that the same factors, such as Bak and Bax, have different rates of proteasome-dependent protein degradation in different cells. Therefore, in either of these scenarios, one of the two opponent groups (i.e., antiapoptotic vs proapoptotic) might have an upper hand in their overall effects. The final balance decides whether the proteasome inhibitor promotes or represses apoptosis.

To better understand the mechanism of how the relative levels of Bik and antiapoptotic Bcl-2 members decide the fate of leukemic cells, we tested the interaction between Bik and Bcl-xL and the consequences of such interaction. We found that Bik coprecipitates with antiapoptotic Bcl-xL in Bcl-xL-transfected NC, and this is a physical basis for them to counteract each other’s effect. Functionally, Bcl-xL overexpression protected apoptosis induced by both proteasome inhibition and by expression of exogenous Bik, suggesting that Bcl-xL and Bik likely interact in the cells. It is conceivable that in wild-type lymphocytes the Bik accumulated after proteasome inhibition will trap more endogenous Bcl-xL, which could normally prevent CytoC release from Mito. The Mito will then be prone to CytoC escape and the freed CytoC will form apotosomes, which activate the downstream caspase pathway reported previously (37, 50). In our model, we do have additional supporting evidence that caspase-3 was activated.

Although the pan-caspase inhibitor zVAD.fmk could inhibit DNA fragmentation, LAC-treated cells eventually died in the presence of zVAD-fmk (data not shown), as has been reported in other apoptosis models (51). This indicates that LAC caused irreversible damage to the cells and the damage was upstream of caspase activation. In all likelihood, the damage is at the mitochondrial level, according to our findings.

Although we have proposed that the augmented level of Bik is sufficient in LAC-induced apoptosis, we are clearly aware that proteasomes can degrade a large number of proteins, some of which might also contribute significantly to the process of apoptosis. There are 6 antiapoptotic and 12 proapoptotic members in the mammalian Bcl-2 family documented so far (33, 52). Conceivably, according to our findings, Bcl-xL overexpression protected apoptosis induced by both cocorticoid-induced apoptosis, and the degradation is a proteasome complex: properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. Biochemistry 32: 1563.


