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Bcl-x_L Overexpression Restricts Heat-Induced Apoptosis and Influences hsp70, bcl-2, and Bax Protein Levels in FL5.12 Cells

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Although several proteins have been identified that can inhibit stress-induced apoptosis, the cytoprotective potential of bcl-x_L against heat shock and its ability to alter hsp70 induction is not known. The current study, using control and *bcl-x_L*-overexpressing IL-3-dependent FL5.12 cells, compared the effects of 1 h of acute heat stress (42 °C) followed by 1, 4, and 8 h recovery (37 °C) on hsp70, bax, bcl-2, and bcl-x_L protein levels and apoptosis. Less than 0.5% of untreated cells were apoptotic. There was significantly more apoptosis in control (~16%) as compared to *bcl-x_L* cells (~3%) 8 h after heat stress. Immunoblotting revealed a time-dependent increase in hsp70 protein levels following 1 h of heat stress in control, but not *bcl-x_L*-overexpressing cells. bcl-2 protein levels were lower in *bcl-x_L*-overexpressing cells than in controls, but decreased in both cell lines after heat stress. bax protein levels in *bcl-x_L*-overexpressing cells were decreased ~80% below baseline levels 1 h post heat shock. This decrease was maintained to 8 h. No change in bax protein was observed in control cells up to 8 h post heat shock. These data indicate that *bcl-x_L* overexpression mitigates the effects of acute heat stress so that hsp70 induction is eliminated and apoptosis is prevented. The rapid loss of bax protein following heat stress in *bcl-x_L*-overexpressing, but not control, cells may contribute to their resistance to apoptosis. Conversely, the loss of bcl-2 protein following heat stress in control cells may contribute to their susceptibility to apoptosis. © 1997 Academic Press

Key Words: **apoptosis; bcl-x_L; bax; hsp70; bcl-2; heat stress; heat shock.**

Apoptosis and necrosis are morphologically and biochemically distinct modes of cell death. Necrosis is associated with inflammation, resulting from a cellular insult that causes cell swelling, mitochondrial malfunctioning, and the release of lysosomal enzymes (1). In contrast, apoptosis, which was originally described for its role in normal homeostatic cell turnover in healthy adult tissues (2), is a form of cell death associated with nuclear and cytosolic condensation and the formation of apoptotic bodies which can be taken up and degraded by neighboring cells (3,4). Apoptosis-associated nuclear condensation is usually accompanied by oligonucleosomal DNA fragmentation into oligomers of ~180 base pairs (5). Failure to regulate apoptosis, which is normally tightly controlled, is associated with autoimmune diseases, congestive heart failure, and myriad neoplastic disorders.

A number of stimuli are reported to initiate apoptosis including, but not limited to, oxidants, various xenobiotics, growth factor withdrawal, glucocorticoid therapy, irradiation, and heat stress (6,7). A family of proteins that is involved in maintaining normal tissue homeostasis by affecting apoptotic cell deletion in both normal and injured/stressed tissues is the BCL-2 family (8). While bcl-2 and bcl-x_L, which share 47% amino acid homology, repress apoptotic cell death, other members of the BCL-2 family—*e.g.*, bax and bcl-x_S—stimulate apoptosis (9). bcl-x_L and bcl-2, 29 and 26 kDa proteins respectively, are found in mitochondria, nuclear membranes, and endoplasmic reticula (9-12). The mechanism by which each protects against apoptosis isn't clear. It was reported, however, that bax (24 kDa) and BCL-2 proteins share homology at putative BH1 and BH2 domains and can form homo- and heterodimers (13). A predominance of bcl-2 homodimers may favor a repression of apoptosis, whereas increased bax levels, relative to bcl-2, potentiates apoptosis. Mixed reports exist as to whether bax can similarly heterodimerize with bcl-x_L, countering bcl-x_L's death repressor activity (14,15).

Heat shock protein 70 (hsp70) is a 70 kDa stress

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protein that can be induced by a variety of stimuli. The precise molecular cytoprotective mechanism of hsp70 isn't understood. It has been suggested that hsp70's cytoprotective mechanism is similar to that of other HSPs in that it behaves as a chaperone by preventing the denaturing and misfolding of proteins (16-18). Others suggest that hsp70's cytoprotective effect may arise from its ability to assist in the transfer of newly synthesized proteins into mitochondria helping to maintain overall mitochondrial integrity (19-21). One recent study indicates that hsp70-mediated cytoprotection involves the prevention of apoptosis by inhibiting caspase activity (7).

Previous studies reported that *bcl-2* and *mcl-1*, a protein with sequence similarity to bcl-2, overexpression affords protection against heat stress (22-26). *bcl-2* expression did not, however, alter the steady-state or heat-induced levels of heat shock proteins as compared to non-expressing cells (22, 23). The present study examined the ability of *bcl-x_L* overexpression in FL5.12 cells (an interleukin (IL)-3 dependent murine prolymphocytic cell line) to protect against heat-induced apoptosis and the induction of hsp70. The results revealed that control cells underwent significantly more heat-induced apoptosis as compared to *bcl-x_L* cells. There was a time-dependent increase in hsp70 in control FL5.12 cells, while in *bcl-x_L* cells hsp70 protein levels were unchanged. Immunoblots against bax revealed a dramatic decrease in this protein in *bcl-x_L* cells post heat shock as compared to control cells. These data indicate that *bcl-x_L* overexpression is cytoprotective against heat-induced apoptosis, and, at the same time, restricts the induction of hsp70.

MATERIALS AND METHODS

Cell culture and heat stress treatment. The murine IL-3-dependent prolymphoid progenitor cell line (FL5.12) transfected to stably express either a SFFV-NEO control construct or an SFFV-FLAG-bcl-x_L construct was used in these studies and has been described previously (15). These cells were acquired originally as a gift from Dr. Gabriel Nuñez (University of Michigan, Ann Arbor). Cells were cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), streptomycin (100 μg/μl) (Gibco, Gaithersburg, MD), and 10% (v/v) IL-3-conditioned medium derived from WEHI-3B cells (39). FL5.12 and WEHI-3B cells were maintained at 37 °C in a 5% CO_2/95% air atmosphere. For heat stress, 1 ml of control and *bcl-x_L*-transfected FL5.12 cells (10^7 cells/ml), along with 3 ml of fresh complete medium (described above), were plated in separate vented 50 ml flasks (Costar, Cambridge, MA) and kept at 42 °C for 1 h. Following heat stress, all flasks were immediately placed at 37 °C for 1, 4, or 8 h of recovery. Cells were pelleted at 200 *g* prior to determining apoptosis or lysis for immunoblots. Cell counts were determined using a T-890 Coulter counter (Coulter, Miami, FL).

Immunoblot assay. Pelleted cells (10^7) were resuspended and lysed in 200 μl of lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) NP-40, 100 μg/ml phenylmethyl-sulfonyl fluoride (PMSF), 30 μl/ml aprotinin and 1 mM sodium orthovanadate] by repeated pipetting, followed by a 20 min incubation on ice. Cell lysates were centrifuged at 16,000 *g* for 10 min to pellet cellular debris and the supernatant was collected and stored at −20 °C. Thawed supernatants were mixed with loading dye [4% (w/v) SDS, 20% (w/v) glycerol, 4% (w/v) β-mercaptoethanol, 0.2 M Tris/HCl (pH 6.8) and 0.02% (w/v) bromophenol blue] and run on either 8 (hsp70) or 15% (BCL-2 family) SDS-polyacrylamide reducing gels. Protein was transferred to PVDF membranes (Millipore, Bedford, MA) and blocked for at least 2 h in 5% (w/v) non-fat dry milk (BioRad, Hercules, CA). Membranes were then incubated with either a polyclonal antibody specific for the inducible form of hsp70 (1:20,000 dilution) (StressGen, Victoria, BC, Canada), polyclonal anti-bax antibody (1:1500 dilution), polyclonal anti-bcl-x_L antibody (1:1500 dilution), or a monoclonal anti-bcl-2 antibody (1:1500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The membranes were rinsed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) (Amersham, Arlington Heights, IL and Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After the secondary antibody incubation, the membranes were rinsed and bound antibodies were detected using enhanced chemiluminescence with a kit from Amersham (Arlington Heights, IL). Individual band densities were integrated using NIH Image public domain software. Immunoblots following the various treatments were performed following a minimum of two independent experiments. Representative blots are shown in the figures. Protein concentrations were measured using the Lowry assay (27).

Apoptosis measurement. Control and *bcl-x_L*-transfected cells (10^6) were plated in fresh complete medium. Following 1 h of heat stress (42 °C), cells were immediately returned to 37 °C for 1, 4, or 8 h recovery. At each recovery time, cells were transferred to microcentrifuge tubes and pelleted at 200 *g* for 10 min. Following centrifugation, the supernatant was discarded and the cell-rich pellets were resuspended in a mixture containing 2 μl of ethidium bromide (100 μg/ml) and 2 μl of acridine orange (100 μg/ml) to assess apoptosis using fluorescence microscopy. Live, early apoptotic, late apoptotic, and necrotic cells were differentiated from each other based on definitive nuclear and cytosolic fluorescence (28). A minimum of 150 cells were counted in 4 random fields per slide. Overall cell viability was also assessed using Trypan Blue exclusion.

Statistics. Data are expressed as means ± S.E. Comparisons between groups were done using a one-way ANOVA followed by a Student-Newman-Keul's test. A *P*-value of less than 0.01 was considered significant.

RESULTS

Less than 0.5% of both control and *bcl-x_L*-overexpressing FL5.12 cells were apoptotic immediately following plating as determined by ethidium bromide/acridine orange (Fig. 1). This level did not change following incubation for up to 8 h at 37 °C (data not shown). Approximately 2.5% of control cells were apoptotic as soon as 1 h of recovery following 1 h of heat stress. No apoptosis above baseline levels was detected in *bcl-x_L* cells at the same time point. At 4 and 8 h post-heat stress, the percentage of apoptotic control cells increased to 9.6 and 16.0%, respectively. *bcl-x_L* transfectants exhibited only 1.3 and 3.0% apoptotic cells following the same recovery times (Fig. 1). Also, control and *bcl-x_L* cells displayed ~89 and 95% overall membrane integrity, respectively, at 8 h of recovery following 1 h of heat stress measured by Trypan Blue (data not shown).

Immunoblot data revealed that the inducible form of hsp70 is expressed constitutively in control and *bcl-x_L*-

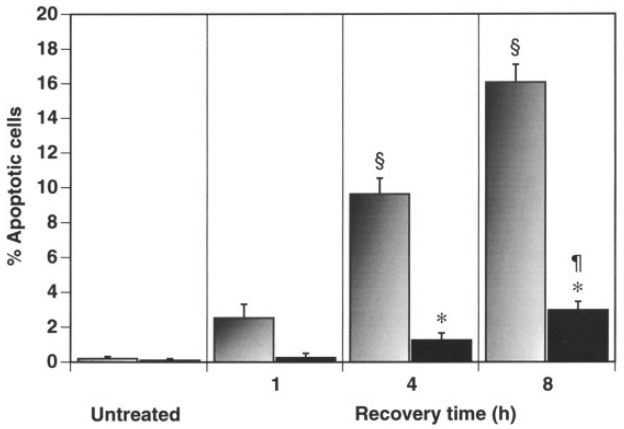

FIG. 1. Apoptosis in untreated and heat-stressed (1 h at 42 °C) FL5.12 cells. Control (▩) and *bcl-x*$_L$-overexpressing (■) cells were plated in fresh complete medium, heat shocked, and analyzed after 1, 4, or 8 h recovery at 37 °C. The percentages of apoptotic cells were assessed using distinct nuclear and cytosolic fluorescence in the presence of acridine orange/ethidium bromide. A minimum of 150 cells in 4 random fields were counted per slide. Results are expressed as means ± S. E. (n=6). §Significantly different from untreated controls. ¶Significantly different from untreated *bcl-x*$_L$ overexpressors. *Significantly different from control counterpart at the same time point. ($P < 0.01$).

transfected FL5.12 cells (Fig. 2, lanes 1 and 5). There was a steady increase to 2-, 3.8-, and 4.8-fold above baseline at 1, 4, and 8 h, respectively, following heat stress in control cells (Fig. 2, lanes 2 to 4). No induction of hsp70 above baseline was observed in *bcl-x*$_L$ cells up to 8 h (Fig. 2, lanes 6 to 8).

Endogenous bcl-x$_L$ protein in control and *bcl-x*$_L$-transfected cells showed a slight decrease by 8 h following heat stress, although this change was likely not meaningful (Fig. 3*A*). No changes in the level of transfected bcl-x$_L$ protein, which contains the FLAG amino acid sequence and thus runs differently on gel electrophoresis, were detected in overexpressing cells up to 8 h after heat stress.

In contrast to the absence of changes in bcl-x$_L$ protein, dramatic changes in bax protein, which has moderate constitutive expression in control and *bcl-x*$_L$ cells, were observed following heat exposure. In particular, *bcl-x*$_L$ cells exhibited an ~80% decrease below baseline levels in bax 1 h after heat shock (Fig. 3*B*, lanes 5 versus 6). This decrease was maintained up to 8 h of recovery (Fig. 3*B*, lane 8). Although containing similar basal levels of bax as *bcl-x*$_L$ cells, heat-shocked control cells exhibited no change in bax protein levels up to 8 h (Fig. 3*B*, lanes 1-4), despite the significant increase in apoptosis (Fig. 1). bcl-2 protein levels in control cells were decreased after heat shock (Fig. 3*C*, lanes 1-4). The somewhat higher level of bcl-2 expression at 4 h (lane 3) was not seen in a repeat experiment where overall bcl-2 expression was less visible. *bcl-x*$_L$-overexpressing cells contained less baseline bcl-2 protein than control cells possibly due to down-regulation in the face of the large levels of bcl-x$_L$ protein (Fig. 3*C*, lanes 5). This expression was decreased to virtually undetectable levels 1 to 8 h following heat shock (Fig. 3*C*, lanes 6 to 8).

DISCUSSION

Numerous intracellular cytoprotective agents—*e.g.*, certain BCL-2 family proteins and hsp70—that can protect against stress-induced apoptosis have been identified. In particular, previous studies have reported on the cytoprotective nature of bcl-2 against heat-induced apoptosis (22-26). Whether bcl-x$_L$, a close relative of bcl-2, can similarly protect against heat-induced apoptosis is not known. The current study used FL5.12 cells transfected to overexpress *bcl-x*$_L$ to examine the ability of this protein to protect against 1 h of acute heat stress. Eight hours was chosen as the maximal recovery time since a previous study reported that following 1 h of heat shock, hsp70 induction increased to ~8 h and then began to decline (29).

A link between hsp70 and the BCL-2 proteins is supported by recent reports indicating that overexpressing either *hsp70* or *bcl-2* in heart muscle provides protection against ischemia-reperfusion-linked cell death, a process that may be more closely linked to apoptosis than previously thought (30-34). Results of the current study showed that FL5.12 cells transfected to overexpress *bcl-x*$_L$ were more resistant than control cells to heat-induced apoptosis. This is consistent with the hypothesis that *bcl-x*$_L$ overexpression protects against and, therefore, mitigates the normal cellular response to heat shock, including apoptosis and induction of hsp70.

hsp70 is an inducible stress protein located in mitochondria, endoplasmic reticula, and cytosol (35) that was originally identified for its ability to enhance thermoresistance to mild hyperthermia (36, 37). Recently,

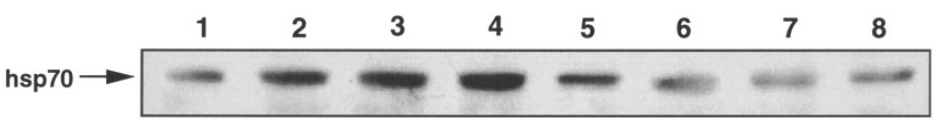

FIG. 2. Immunoblot analysis of hsp70 in heat-stressed FL5.12 cells. Cells were heated for 1 h at 42 °C, followed by 1, 4, or 8 h recovery at 37 °C. Lanes 1-4 and 5-8 represent untreated, 1, 4, and 8 h recovery in control and *bcl-x*$_L$ cells, respectively. Each lane was loaded with 20 μg of total protein and an antibody specific for the inducible form of hsp70 was used.

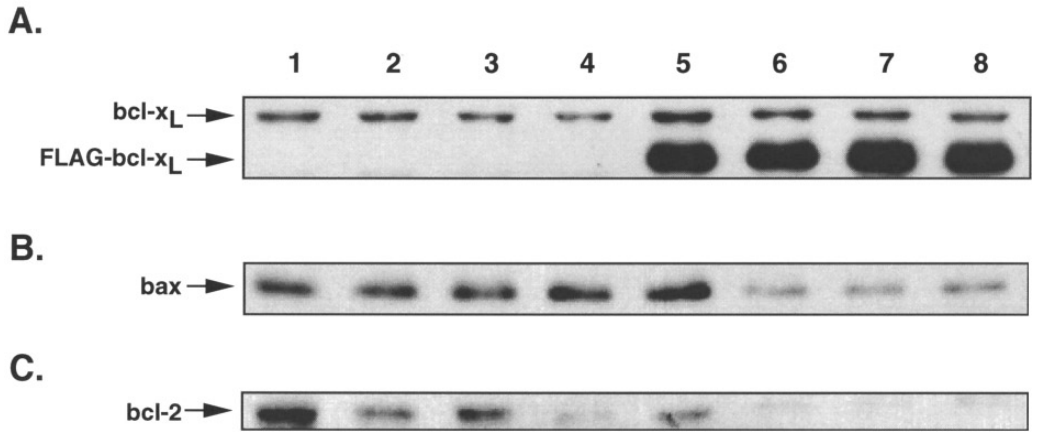

FIG. 3. Immunoblot analysis of (**A**) bcl-x_L, (**B**) bax, and (**C**) bcl-2 proteins in heat-stressed FL5.12 cells. Cells were heat stressed for 1 h at 42 °C, followed by 1, 4, or 8 h recovery at 37 °C. Lanes 1-4 and 5-8 represent untreated, 1, 4, and 8 h recovery in control and *bcl-x*$_L$ transfectants, respectively. Each lane was loaded with 20 μg (**A** and **B**) or 40 μg (**C**) of total protein. The anti-bcl-x_L antibody detects both the native and transfected form of bcl-x_L. The transfected form contains the FLAG amino acid sequence resulting in a different migration pattern in the gel.

it was reported that cleavage of the DNA repair enzyme, poly (ADP-ribose) polymerase (PARP), by caspase-3 is inhibited in *hsp70*-overexpressing cells exposed to acute heat stress (7). Similarly, bcl-2 and bcl-x_L proteins inhibit staurosporine-induced apoptosis, at least in part, by maintaining caspase-3 in its inactive form (38). Other studies showed that exposure of *bcl-2*-expressing cells to a thermotolerance-promoting pretreatment of heat stress enhanced these cells' resistance to apoptosis (22,23). Taken together, these reports suggest that hsp70 and BCL-2 proteins may act separately in preventing apoptosis since each protein effectively restricts apoptosis but can have synergistic anti-apoptotic effects when they are present together.

While the anti-apoptotic mechanisms of BCL-2 proteins and hsp70 may be independent, the current study revealed that *bcl-x*$_L$ transfectants, in addition to being more resistant to heat-induced apoptosis, showed no hsp70 induction above baseline. Control cells, in contrast, experienced a steady increase in this protein up to 8 h following heat stress. The increase in hsp70 in control cells, however, may not have been rapid enough to protect these cells from undergoing apoptosis. These data, along with the results from other investigators, suggest that cells must either constitutively express high levels of hsp70 or be exposed to a thermotolerance-promoting pretreatment, in order to be protected by this protein against heat-induced apoptosis (7,29). Our data suggest that bcl-x_L, unlike bcl-2 that does not inhibit the induction of heat shock proteins (22,23), may be able to regulate hsp70 protein levels; perhaps by acting upstream of hsp70, restricting its synthesis. Alternatively, differences in cell types might explain these differing results or it might be that bcl-x_L is more effective than bcl-2 at mitigating the cellular stress response which includes hsp70 production. In other words, *bcl-x*$_L$ overexpression may prevent hsp70 induction because these cells aren't being stressed or the effects of the stress aren't manifested in a way that stimulates hsp70 production.

An opposing interpretation to hsp70 induction occurring too late to protect 16% of control cells from undergoing apoptosis is that hsp70 induction is actually cytoprotective. It may be that hsp70 is protecting the other 84% of cells from undergoing apoptosis and if hsp70 induction were blocked, then far more than 16% of control cells would undergo heat-induced apoptosis.

Large changes in bax protein were detected following heat shock. bax, also a member of the BCL-2 family, promotes apoptosis by heterodimerizing with and antagonizing the death suppressor activity of bcl-2 (13). Whether bax can similarly heterodimerize with and counter the death suppressor activity of bcl-x_L remains unclear (14,15). What is accepted, however, is that heterodimerization with bcl-x_L is not required for bax to counter its death suppressor activity (15). The dramatic ~80% decrease in bax protein in *bcl-x*$_L$ transfectants up to 8 h after heat stress, correlates well with these cells' resistance to apoptosis. The mechanism that mediates this loss solely in *bcl-x*$_L$-transfected cells is not known. bcl-2 protein was lost from both control and *bcl-x*$_L$-overexpressing cells. Although this might be expected to counter the loss of bax, the overwhelming level of bcl-x_L protein in transfected cells may have obscured any such effect.

In summary, *bcl-x*$_L$ overexpression in FL5.12 cells lessens the effects of acute heat shock so that hsp70 induction and apoptosis are restricted compared to controls. Furthermore, bax levels are greatly reduced following a heat stress in *bcl-x*$_L$-overexpressing cells, an effect that may contribute to their resistance to

apoptosis. Conversely, the loss of bcl-2 protein following heat stress in control cells which lack the countering effect of excess bcl-x_L protein may contribute to their susceptibility to apoptosis.

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