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Protection of hybridoma cells against apoptosis by a loop domain-deficient Bcl-xL protein

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Abstract

The ectopic expression of several members of the Bcl-2 family of anti-apoptotic proteins is a promising strategy to improve the viability of hybridoma cells in culture. However, the impact of post-translational modifications on the function of these proteins in murine hybridomas is unknown. To address this issue, the anti-apoptotic properties of a mutant of Bcl-xL devoid of the so-called ''loop domain'' (Bcl-xLΔ46-83) were investigated using the Sp2/O-Ag14 hybridoma model. Clones of Sp2/O-Ag14 cells expressing Bcl-xLΔ46-83 exhibited resistance against L-glutamine deprivation to similar levels than cells expressing the wild type protein. In contrast, protection against the cytotoxic effects of cycloheximide (CHX) was highly dependent on the level of expression of the Bcl-xLΔ46-83 mutant. Analysis of the growth behaviour of the transfected cells showed that Bcl-xLΔ46-83 was superior to the wild type protein in prolonging Sp2/O-Agl4 cell viability in stationary batch culture. Furthermore, the prolongation of cell viability in batch culture was directly proportional to the level of expression of the mutated protein. Our results indicate that removal of the loop domain improves the anti-apoptotic activity of Bcl-xL in hybridoma cells grown in stationary batch culture.

Abbreviations: CHX – cycloheximide, L-Gln – L-glutamine, HA – Haemagglutinin epitope, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS – Phosphate-buffered saline, SDS – Sodium dodecyl sulphate

Introduction

Cell death by apoptosis is a major factor limiting the viability, and by consequence the productivity, of murine hybridomas and other mammalian cell lines of biotechnological interest (Al-Rubeai and Singh 1998; Cotter and Al-Rubeai 1995). Stresses such as nutrient limitation, anoxia and hydrodynamic stress have been shown to be the principal triggers of cell death in batch or perfusion hybridoma cell cultures (Franek 1995; Mercille and Massie 1994a, 1994b; Singh et al. 1994). Intense efforts have been made in order to find ways to delay the induction of apoptosis in hybridoma cell lines, with the expectation that this will result in increased monoclonal antibody productivity.

One strategy that may prove effective in improving hybridoma cell viability is the ectopic expression of anti-apoptotic genes of the Bcl-2 family. Several groups have reported increases in hybridoma viability in stationary batch culture following the overexpression of Bcl-2 (Fassnacht et al. 1998; Itoh et al. 1995; Simpson et al. 1997; Terada et al. 1997), Bcl-xL (Charbonneau and Gauthier 2000) and the adenovirus Bcl-2 homologue E1B-19K (Mercille et al. 1999). However, the extent of the increase in viability resulting from the overexpression of Bcl-2 proteins is

variable and clearly dependent on the cellular context. For example, while the expression of Bcl-2 in the NS/O myeloma failed to prolong cell survival (Fujita et al. 1997; Murray et al. 1996), EIB-19K markedly increased the viability of this cell line (Mercille et al. 1999). These observations suggest that regulatory mechanisms targeting specific Bcl-2 family members are operative in murine hybridoma and myeloma cell lines. Such mechanisms may include phosphorylation, ubiquitin-mediated proteolysis, caspase-mediated cleavage, protein-protein interactions and changes in sub-cellular localisation (Fadeel et al. 1999).

The determination of the tertiary structure of Bcl-xL revealed the presence of a long, unstructured region located between the α1 and α2 helices (Muchmore et al. 1996). This ''loop domain'', spanning amino acids 26 to 83 in Bcl-xL, has been shown to be the site of several post-translational modifications affecting the activity of both Bcl-xL and Bcl-2. For example, interleukin-3 or erythropoietin treatment of NSF/N1.H7 cells induced the phosphorylation of Ser 70, resulting in the inactivation of Bcl-2 and subsequent apoptosis (Ito et al. 1997). Similarly, dephosphorylation of residues Thr 56, Thr 74, and Ser 87 has been linked to the rapid ubiquitination and proteosome-mediated degradation of Bcl-2 (Dimmeler et al. 1999). Caspase mediated cleavage of Bcl-2 and Bcl-xL has been shown to occur in the loop domain, giving rise to fragments with a marked pro-apoptotic activity (Cheng et al. 1997; Fujita and Tsuruo 1998). Interestingly, removal of the loop domain of Bcl-2 or Bcl-xL usually results in a protein with greater anti-apoptotic properties, indicating that this region acts as a negative regulatory domain.

In order to examine the relative contribution of post-translational modifications on the function of anti-apoptotic Bcl-2 proteins in murine hybridomas, we transfected Sp2/O-Ag14 cells with a Bcl-xL mutant lacking the loop domain. Cells expressing either the loop domain deficient mutant or the wild type Bcl-xL were tested for resistance to apoptosis induced by L-glutamine (L-Gln) deprivation or exposure to the protein synthesis inhibitor cycloheximide (CHX). Cell growth behaviour in stationary batch culture was also examined.

Materials and methods

Reagents

Unless otherwise stated, all reagents were obtained form Canadian Life Technologies (Burlington, ON).

Cell line and cell maintenance

The murine hybridoma Sp2/O-Agl4 (ATCC CRL 1581) was obtained from the American Type Tissue Collection (Rockville, MD) and maintained in Iscove's modified Dulbecco's media (Princess Margaret Hospital, Toronto, ON) supplemented with 5 % Fetalclone I (Hyclone, Logan, UT), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cell culture was performed in 25 cm^2 flasks, at 37 °C under an atmosphere of 5% CO_2/95% air.

Plasmid contructs and cell transfection

A mutant of Bcl-xL (Bcl-xL Δ46-83) devoid of the loop domain was obtained following a two-step PCR procedure (Pont-Kingdon 1994). The 5′ mutagenesis primer was as follows (numbering refers to the position in the open reading frame of Bcl-xL): G_{115}AAGAAACTGAAGCAGAGAGGG$_{136}$**CTGCTGCTGCTG**C_{251}AGCAGTGAAGCAAGAGATG-$_{270}$. The resulting mutation replaces amino acids 46 to 83 of Bcl-xL, which correspond to part of the loop domain, by four alanine residues (indicated in bold in the sequence). This mutant has been shown by others to possess increased anti-apoptotic activity (Chang et al. 1997). A hemagglutinin tag (YPYDVPDYA) was introduced at the C-terminal of both the mutated and wild type Bcl-xL. Finally, a Kozak consensus sequence (ACCACC) was placed immediately upstream of the initiation codon of both cDNAs. Both the loop domain-deficient and the wild type cDNAs were sequenced (Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON) and cloned in the mammalian expression vector pTEJ8 (a kind gift of Dr. Réal Lemieux, Héma-Québec, Ste-Foy). Transfection of Sp2/O-Agl4 cells was carried out by electroporation (Gene Pulser Plus; Bio-Rad, Mississauga, ON) as described previously (Charbonneau and Gauthier 2000). Selection of stably transfected cells was performed by the inclusion of geneticin (750 μg ml^{-1}) in the culture media.

Protein extract preparation and western analysis

Soluble protein extracts were prepared in RIPA buffer (1% Igepal [Sigma], 1% sodium deoxycholate [Sigma] and 0.1% SDS) in PBS (9.1 mM Na_2HPO_4, 1.7 mM NaH_2P0_4, 150 mM NaCl, pH 7.4) as described previously (Charbonneau and Gauthier 2000). The extracts were stored at −80 °C until needed. Proteins were fractionated on a 12% PAGE-SDS gel,

transferred onto a Hybond-P membrane (Amersham-Pharmacia Biotech, Baie-d'Urfé, QC), and processed for Western analysis using rabbit polyclonal anti-HA (Y-11) or anti-Bcl-xL (S-18) primary antibodies and a goat anti-rabbit IgG secondary antibody coupled to horseradish peroxidase (all from Santa Cruz Biotechnology Inc, Santa Cruz, CA). Detection was performed using the ECL-Plus Chemiluminescence kit and Hyperfilm-ECL autoradiography film (Amersham Pharmacia), as instructed by the manufacturer. Quantification of the relative abundance of the Bcl-xL-HA and Bcl-xL Δ46-83-HA proteins was carried out using the AlphaEase software (Alpha-Innotech, San Leandro, CA).

MTT assay

Cells (1×10^5) were incubated in a total volume of 100 μl in 96-well plates in the presence or absence of 25 μg ml^{-1} CHX (Sigma). Alternatively, cells were incubated in Iscove's media lacking L-Gln (Sigma). Cells were incubated at 37 °C tor 3 h (CHX) or 4 h (L-Gln deprived) before the addition of 25 μl of MTT dye (Sigma; 5 mg ml^{-1} dissolved in PBS). Two h later, 100 μl of lysis buffer (20% SDS in 50% N-N′-dimethylformamide, pH 4.7) was added to each well and the plates were incubated at 37 °C overnight. Optical density readings were performed at 570 nm using a PowerWaveX microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Results are expressed as the average ± standard deviation of three independent experiments.

DNA fragmentation

Five hundred thousand cells were collected and washed once with PBS. The cell pellet was lysed with 50 μl of lysis buffer (10 mM Tris-HCI pH 8, 1 mM EDTA pH 8, 0.5% N-lauroyl sarcosine, 0.25 mg ml^{-1} proteinase K and 0.02 mg ml^{-1} RNAse A) and incubated for 5 min at 50 °C. Fifty microliters of sample buffer (40% sucrose, 0.08% bromophenol blue) was then added to the sample, followed by electrophoresis on a 2% agarose gel in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA pH 8). The DNA was then stained for 5 min with ethidium bromide (0.7 μg ml^{-1}) and visualised under UV illumination.

Stationary hatch culture

Exponentially growing cells were centrifuged and resuspended in fresh culture media at a density of 5×10^4 cells ml^{-1} in 25 cm^2 flasks and cultured as described above. Cell viability was determined daily using the trypan blue exclusion assay: an aliquot of cells was diluted in 0.04% trypan blue dye (Sigma) dissolved in PBS. The viable (membrane-intact) and dead cells were then counted using a Neubauer hemacytometer. Each result is the average ± standard deviation of at least 4 determinations.

Results

Expression of a loop domain-deficient mutant of Bcl-xL in Sp2/O-Ag14 cells

Three Sp2/O-Ag14 clones stably expressing the loop domain-deficient Bcl-xL (Sp2Δ46-83) and two clones expressing wild-type Bcl-xL (Sp2-Bcl-xL) were obtained and analysed by Western analysis (Figure 1). Using an anti-HA antibody, we found that the three Sp2Δ46-83 clones expressed increasingly higher amounts of the mutant protein, in ratios of approximately 1:2:4 (Figure 1A). We therefore labelled these clones Sp2Δ46-83^{low}, Sp2Δ46-83^{med}, and Sp2A4Δ83^{high}, to reflect the relative levels of expression of the mutated Bcl-xL protein in these cells. The expression level of each of the loop domain-deficient Bcl-xL was considerably higher than the wild type protein, with the Sp2Δ46-83^{low} clone expressing approximately 4 fold more Bcl-xL than the Sp2-Bcl-xL clones.

The wild-type Bcl-xL protein appeared as a doublet of closely migrating bands, which was most evident when an anti-Bcl-xL antibody was used (Figure 1B). This doublet has been ascribed as post-translational modifications, most likely phosphorylation, affecting the Bcl-xL protein (Chang et al. 1997; Haldar et al. 1996). Interestingly, the loop domain-deleted mutants migrated as a sharp, single band, suggesting that the modifications targeting the wild type form of Bcl-xL did not occur on the mutated protein. As described previously (Charbonneau and Gauthier 2000), the vector-containing cells (Sp2-pTEJ8) had undetectable amounts of the endogenous Bcl-xL protein.

The extent of cell death inhibition by the BcI-xLΔ46-83 mutants varies depending on the stimuli

Amino acid deprivation is an important factor limiting the viability of hybridoma cultures (Mercille and

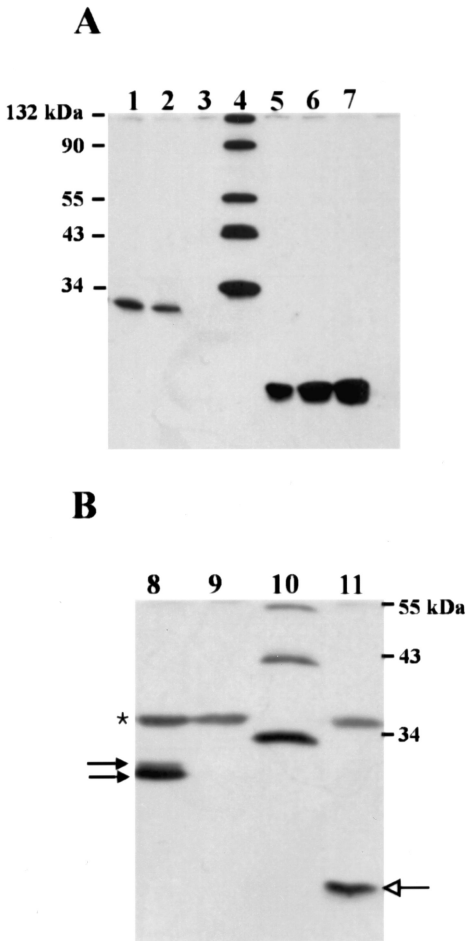

Figure 1. Western blot analysis. The expression of HA-tagged Bcl-xL or Bcl-xLΔ46-83 in Sp2/O-Ag14 cells was analysed using an anti-HA antibody (A) or an anti-Bcl-xL antibody (B). Lanes 1 and 8: Sp2-Bcl-xL clone 14; lane 2: Sp2-Bcl-xL clone 4; lanes 3 and 9: Sp2-pTEJ8; lane 4 and 10: Cruz Molecular Weight marker; lane 5 and 11: Sp2Δ46-83^{low}; lane 6: Sp2Δ46-83^{med}; lane 7: SP2Δ46-43^{high}. The closed arrows indicate the Bcl-xL doublet. The open arrow shows the loop-deficient Bcl-xL protein. The star designates a protein cross-reacting with the Bcl-xL antibody.

Massie 1994a; Simpson et al. 1998). We therefore tested the ability of the mutated Bcl-xL protein to protect against the loss of cell viability that accompanies L-Gln deprivation. In agreement with our previous observations (Charbonneau and Gauthier 2000), the wild type Bcl-xL significantly protected Sp2/O-Ag14 cells against apoptosis under conditions of L-Gln limitation, while the pTEJ8-transfected control cells underwent cell death (Figure 2). Expression of the loop domain-deleted Bcl-xL protein was also efficient in maintaining Sp2/O-Ag14 cell viability, however, the level of protection afforded by Bcl-xLΔ46-83 was similar to Bcl-xL. In addition, the level of protection afforded by Bcl-xLΔ46-83 was similar for the Sp2Δ46-83^{low} and Sp2Δ46-83^{high} clones, indicating that Bcl-xL is saturating under these conditions.

To further test the potential of the loop domain-deleted Bcl-xL protein to protect against apoptosis, we exposed our transfected cells to CHX, a protein synthesis inhibitor previously shown to rapidly induce apoptosis in the Sp2/O-Ag14 cell line and related hybridomas (Gauthier et al. 1996; Perreault and Lemieux 1993). As shown in Figure 3A, the control cells rapidly died in the presence of CHX, while expression of the wild-type Bcl-xL significantly protected against cell death. DNA fragmentation analysis confirmed that the cells died by apoptosis (Figure 3B). Expression of the mutated Bcl-xL protected Sp2/O-Ag14 cells against CHX-induced apoptosis. However, in contrast to the L-Gln depletion data (Figure 2), the extent of the inhibition was clearly a function of the expression levels of the Bcl-xLΔ46-83 protein (Figure 3A and B). Effectively, even though it expressed higher levels of Bcl-xL protein, the Sp2Δ46-83^{low} clone showed a greater sensitivity to the cytotoxic effect of CHX that the Sp2-Bcl-xL cells (Figure 3A). On the other hand, both Sp2Δ46-83^{med} and Sp2Δ46-83^{high} clones were at least as resistant as the SP2-Bcl-xL clone towards CHX-induced cell death (Figure 3A). These results indicate that, while the Bcl-xLΔ46-83 protein is effective in inhibiting CHX-induced apoptosis in Sp2/OAg14 cells, the removal

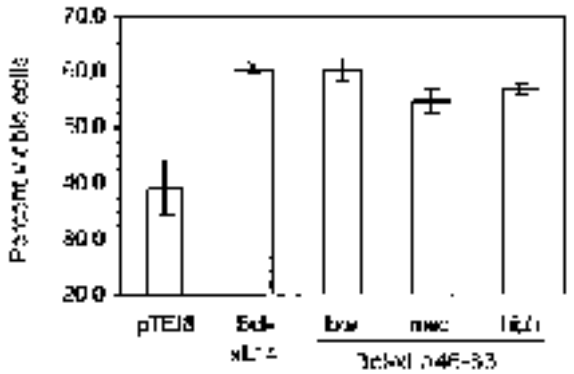

Figure 2. Effect of Bcl-xLΔ46-83 on the survival of Sp2/O-Ag14 cells following L-Gln deprivation. Sp2/O-Ag14 cells transfected with the indicated plasmids were cultured in Iscove's media with or without L-Gln for 4 h. Cell viability was then determined using the MTT assay. Results are expressed as percent cell viability after L-Gln deprivation. Identical results were obtained for both Bcl-xL-expressing clones.

of the loop domain significantly decreased the specific activity of the Bcl-xL molecule. They also suggest that the level of protection afforded by Bcl-xLΔ46-83 varies depending on the apoptotic stimuli.

Culture behaviour of the Sp2Δ46-83 clones

We next determined the effect of the mutated Bcl-xL protein on the viability of hybridoma cells under prolonged culture conditions. The pTEJ8-transfected cells behaved as expected in stationary batch culture (Charbonneau and Gauthier 2000), with a maximal viable cell density of 14.4×10^5 cells ml^{-1} reached on culture day 4 (Figure 4). In agreement with our previous observations (Charbonneau and Gauthier 2000), the Bcl-xL transfected cells showed increased viability in stationary batch culture, with a maximal viable cell density of $21{,}3 \times 10^5$ cells ml^{-1} attained on day 6. Each of the Sp2Δ46-83 clones also showed

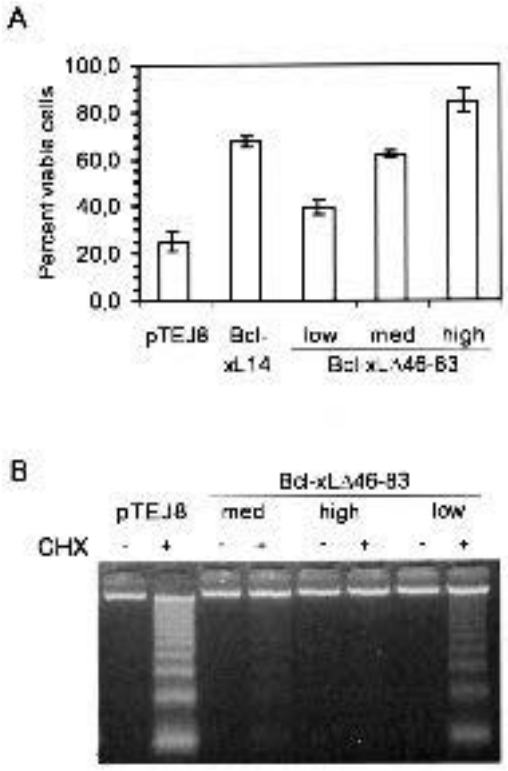

Figure 3. Effect of Bcl-xLΔ46-83 on the survival of Sp2/O-Ag14 cells exposed to CHX. Sp2/O-Ag14 cells transfected with the indicated plasmids were cultured in the presence (+) or absence (−) of CHX for 3 h. MTT cell viability assay (A) and DNA fragmentation analysis (B) were then carried out as described in Materials and Methods. Identical results were obtained for both Bcl-xL-expressing clones.

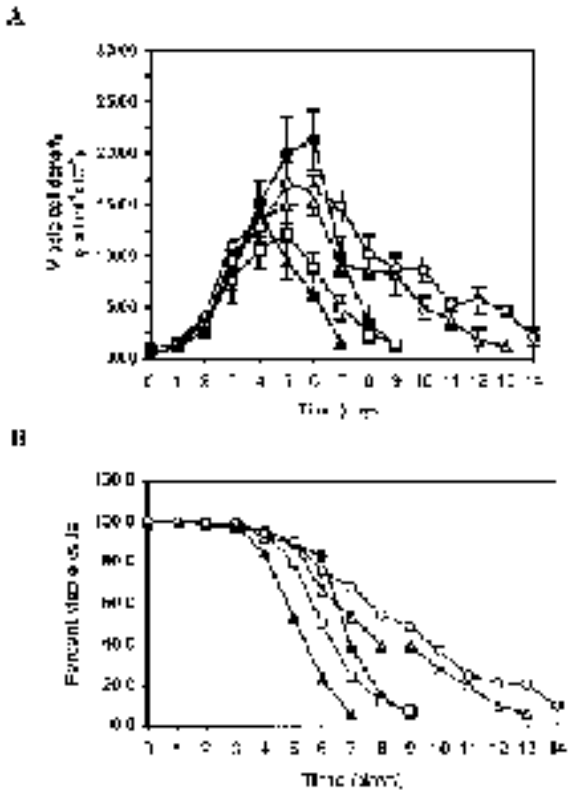

Figure 4. Culture behaviour of transfected Sp2/Q-Ag14 cells. Cells were seeded on day 0 and cell viability was determined daily using the trypan blue dye exclusion assay. (A) Viable cell density. (B) Percentage of viable cells in the culture. Closed triangles: Sp2-pTEJ8; closed circles: Sp2-Bcl-xL, clone 14; open squares: Sp2Δ46-83^{low} open triangles: SP2Δ46-83^{med} open circles: Sp2Δ46-83^{high}.

improved viability under prolonged culture conditions (Figures 4A and B). Remarkably, the extent of the increase in Sp2/O-Ag14 cell survival, as well as the maximal density of viable cells, was directly proportional to the level of expression of Bcl-xLΔ46-83 (Figures 4A and B). Most importantly, the Sp2Δ46-83^{med} clone, which was as resistant as the Sp2-Bcl-xL cells to the cytotoxic action of CHX (Figure 3), exhibited a significantly greater survival in stationary batch culture than the cells expressing the wild type protein.

Discussion

When compared to the wild type Bcl-xL, the Bcl-xLΔ46-83^{med} mutant was superior at improving cell viability under prolonged culture conditions. Effec-

tively, the total culture time for this clone (defined here as the time required to achieve less than 5% viability) was doubled compared to the pTEJ8-transfected cells. This effect was not related to the greater expression levels of the loop domain-deleted protein, since the Bcl-xLΔ46-83^{med} clone was as effective as Bcl-xL in protecting Sp2/O-Ag14 cells against two apoptotic stresses (CHX exposure and L-Gln deprivation) (Figure 2,3). This clearly shows that the mutagenesis of Bcl-2 proteins can improve their potency in B cell hybridomas, leading to greater cell survival in stationary batch culture. Our results therefore confirm and extend those of Figueroa et al. (2001), who have recently showed that a loop domain-deficient Bcl-2 molecule lead to a greater protection of CHO and BHK cells against growth factor withdrawal.

Interestingly, while our Bcl-xLΔ46-83 clones showed enhanced viability in batch culture, they behaved similarly to the Bcl-xL expressing cells when cultured in the absence of L-Gln. This suggests that, while L-Gln exhaustion constitutes an important trigger of apoptosis in murine hybridoma cultures (Mercille and Massie 1994a; Simpson et al. 1998), other stresses participate to limit cell viability. According to our data, such stresses could be distinguished from L-Gln starvation by their greater response to the inhibitory effects ot Bcl-xL. Interestingly, the extent of the increase in cell viability in batch culture closely mimicked the relative resistance of each clone to CHX induced apoptosis, indicating that the molecular events triggered by the culture stresses may share some of the signalling mechanisms responsible for the cytotoxic effect of CHX.

The ability of the Bcl-xLΔ46-83 mutant to protect against apoptosis in the murine hybridoma Sp2/O-Ag14 cell line was dependent on the type of death stimuli. Effectively, both the mutated Bcl-xL and the wild type protein were equally efficient in improving the survival of Sp2/O-Ag14 cells following L-Gln deprivation, and this effect did not depend on the expression levels of the loop domain-deleted mutant (Figure 2). In marked contrast, protection against the cytotoxic effect of CHX was directly proportional to the expression levels of Bcl-xLΔ46-83 (Figure 3). In either case, however, the extent of apoptosis inhibition was clearly lower than what should be expected solely from the expression levels, indicating that the removal of the loop domain reduced the specific activity of Bcl-xL in our system. A similar situation has been observed in HL-60 cells, where the removal of amino acids 32-80 of the loop domain of Bcl-xL resulted in a decrease in the protection against apoptosis induced by Taxol but not ara-C or etoposide (Fang et al. 1998). This may be caused by the presence, in the loop domain, of amino acid residues that play a positive role in the function of Bcl-xL. For example, mutating Ser 70 to an alanine residue yields an unphosphorylated Bcl-2 protein with decreased anti-apoptotic properties (Yokote et al. 2000). In contrast, mutation of residues Thr 56, Thr 74, and Ser 87 of Bcl-2 prevented its degradation via the ubiquitin pathway, leading to an increased cellular resistance to apoptosis (Dimmeler et al. 1999). It will be interesting to determine whether Bcl-xL proteins that have been mutated at selected amino acid residues to prevent specific post-translational modifications without affecting others could be even more useful in increasing the resistance of hybridoma cells to culture stresses.

In conclusion, the data presented in this report demonstrate that the mutagenesis of Bcl-xL can lead to greater improvements in the viability of biotechnologically relevant cell lines in batch culture. Further studies are required in order to determine whether specific point mutations of Bcl-2 proteins could be more advantageous, as well as to examine the effect of the mutagenized proteins on antibody productivity. Finally, our results stress the importance of using appropriate apoptotic stimuli when investigating the relative effectiveness of anti-apoptotic Bcl-2 proteins in murine hybridomas.

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