The Super Anti-apoptotic Factor Bcl-xFNK Constructed by Disturbing Intramolecular Polar Interactions in Rat Bcl-xL*

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A powerful artificial anti-apoptotic factor will be useful for medical applications of the future therapies for many diseases by prolonging survival of sick cells. For constructing it, we designed the super anti-apoptotic factor by disturbing three intramolecular polar interactions among α-helix structures of Bcl-xL. The resultant mutant Bcl-xL, named Bcl-xFNK, was expected to make the pore-forming domain more mobile and flexible than the wild-type. When overexpressed in Jurkat cells, Bcl-xFNK was markedly more potent in prolonging survival following apoptosis-inducing treatment with a kind of cell death cytokines (anti-Fas), a protein kinase inhibitor (staurosporine), cell cycle inhibitors (TN-16, camptothecin, hydroxyurea, and triochostatin A), or oxidative stress (hydrogen peroxide and paraquat) than wild-type Bcl-xL. Furthermore, the transfectants of bcl-xFNK became more resistant against a calcium ionophore and even a heat treatment than wild-type Bcl-xL. In addition, Bcl-xFNK showed marked anti-apoptotic activity in Chinese hamster ovary and Jurkat cells deprived of serum. Thus, Bcl-xFNK may be the first mutant generated by site-directed mutagenesis of Bcl-xL with a gain-of-function phenotype. Interestingly, Bcl-xFNK was found to allow interleukin-3-dependent FDC-P1 to grow without interleukin-3, but not Ba/F3. In Bcl-xFNK transfectants of FDC-P1 and Jurkat, the p42/p44 mitogen-activated protein kinase was activated by 2 to 5 times, but not in those of Ba/F3 and Chinese hamster ovary. Bcl-xFNK might gain a new function to activate the mitogen-activated protein kinase in a cell-type specific manner. The findings of this study suggest that the central α5-α6 pore-forming region of anti-apoptotic factor Bcl-xL has a pivotal role in suppressing apoptosis.

Apoptosis plays an important role in a wide variety of physiological and pathological processes in multicellular organisms. Inhibition of excess apoptosis would be an important strategy of the future therapies for many diseases. As regulators of apoptosis, the bcl-2 family consists of anti-apoptotic and pro-apoptotic factors. Bcl-xL, a member of the bcl-2 family, strongly protects cells from apoptosis (1). Muchmore et al. (2) determined the crystal structure of human Bcl-xL that had been prepared through denaturing and refolding and have proposed that Bcl-xL forms an ion channel based on the similarity in three-dimensional structure between human Bcl-xL, diphtheria toxin, colicin A, and exotoxin. As expected, Bcl-xL was shown to form the ion channel in synthetic lipid membranes, but at a non-physiological pH (3). However, the mechanism by which the ion-channel activity of Bcl-xL associates with its anti-apoptotic effect is not clear. Several mutagenesis experiments have been performed to clarify the relationship between the channel activity and the apoptosis-regulating function (4–7). However, there is no direct evidence that the Bcl-2 family proteins regulate apoptosis based on the ion channel activity in vivo, and the mechanism by which the ion channel activity is associated with their opposing functions in apoptosis regulation is unknown. It is important to clarify how the α5-α6 region of the Bcl-2 family proteins participates in apoptosis regulation.

We have independently crystallized native rat Bcl-xL to determine the structure with a 2.5-Å resolution and found that Bcl-xL has nine intramolecular polar interactions to stabilize the central pore-forming domain (8). It is noted that the refined structure is valuable and useful to design an artificial factor with a powerful anti-apoptotic activity as well as to investigate the molecular mechanism of the Bcl-2 family proteins.

To construct the powerful super anti-apoptotic factor, we focused on the intramolecular interactions between the central pore-forming domain (α5-loop-α6) and the other regions, and designed to change amino acid residues involved in hydrogen bonds in Bcl-xL by site-directed mutagenesis. As expected, the strongest anti-apoptotic factor was constructed by changing three amino acid residues.

MATERIALS AND METHODS

Cells and Culture—Chinese hamster CHO* KI cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (Life Technologies) containing 10% fetal bovine serum. A murine promyeloyeld cell line FDC-P1 and a human leukemia cell line Jurkat were cultured in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum. For IL-3-dependent FDC-P1, IL-3 was added in the medium.

Plasmid Constructs and Transfection—A rat bcl-x cDNA was cloned into mammalian expression vector pEF1BOS (9) to obtain pEF1BOSbcl-x (10). To express mutant Bcl-xFNK in cells, amino acid substitutions were introduced into Bcl-xL by a two-step polymerase chain reaction mutagenesis method using pEF1BOSbcl-x as a template (11). The substituted codons were as follows; Tyr22 (TAC) with Phe (TTC), Gln26 (CAG) with Asn (AAC), and Arg165 (CGG) with Lys (AAG). The coding region of pEF1BOSbcl-xFNK was sequenced to confirm that the mutant Bcl-xFNK does not have any extra mutations. By the same mutagenesis method, other Bcl-xL mutants, Bcl-xL(A103S) (TCT) to Ala (GCT), Asn5 (AAC) to Gln (CAA), Bcl-xFD (Tyr12 (TAC) to Phe (TTC),

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Glutamate (Glu) residues (Glu124, Glu126, Ser164) and Arg165 were replaced with Asp, Ala, and Lys, respectively (AAT to CGG, CAG to CGG, and CGG to CAG). The resulting Bcl-xFNK was overexpressed in Jurkat and FDC-P1 cells, with up to 10-fold higher expression levels compared to wild-type Bcl-xL. The Bcl-xFNK showed enhanced anti-apoptotic activity compared to wild-type Bcl-xL, as demonstrated by the increased survival rate of Jurkat cells treated with chemotherapeutic agents such as etoposide, paraquat, or A23187 (Calbiochem) for 3 to 4 days without medium exchange. The surviving cells were counted daily by trypan blue exclusion method everyday. CHO cells were plated at a density of 1000 cells/100-mm dish in RPMI 1640 without fetal bovine serum at 1 x 10^5 cells/ml and incubated at 37 °C. The plates were removed for every third day, and half of the medium was exchanged with fresh medium. The remaining medium was harvested and used for the assay.

Cell-cycle phase distributions of the cells were determined with a flow cytometer, EPICS ELITE ESP (Coulter, Flolida) for 24 h. The surviving cells were counted by the trypan blue exclusion method everyday. CHO cells were plated at a density of 1000 cells/100-mm dish in RPMI 1640 without fetal bovine serum at 1 x 10^5 cells/ml and incubated at 37 °C. The plates were removed for every third day, and half of the medium was exchanged with fresh medium. The remaining medium was harvested and used for the assay.

MAP Kinase Activity—BIOTRAK p24/p44 MAP kinase enzyme assay system (Amersham Pharmacia Biotech) was used to measure the p24/p44 MAP kinase activity of cells. According to the protocol of the supplier, washed cells were lysed in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, and 1 mM orthovanadate, pH 7.4, containing protease inhibitor mixture (Sigma), and centrifuged to obtain the supernatant retaining cytoplasmic MAP kinases. After a 30-min phosphorylation reaction, phosphorylated substrate was separated using a peptide-binding paper disc. After a wash, the paper disc was analyzed with the fluoro bioimaging analyzer, FLA-2000. The radioactivity on the paper was measured and normalized with the protein amount in the supernatant used for the assay.

RESULTS

Rationale for Construction of Bcl-xFNK—We found 17 intramolecular interactions among a-helix structures to stabilize Bcl-xL, as shown by x-ray structure analysis (8). Fig. 1A shows backbones of Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydrogen bond to stabilize the a-helix structures to stabilize Bcl-xL, in which two amino acid residues involved in each hydro
respectively (Fig. 3I). In addition, Northern blot analysis indicated the same mRNA level in the transfectants (data not shown).

Fig. 2 shows that Bcl-xFNK has a stronger anti-apoptotic activity than Bcl-xL against cell cycle inhibitors as apoptosis-inducing reagents (Fig. 2, A-D). To confirm that TN-16 functioned inside the cell, the status in the cell cycle was examined by the flow cytometric technique. As shown in Fig. 2E, Jurkat-bcl-xL cells were arrested in the M phase as were Jurkatvec and Jurkat-bcl-x cells, indicating that Bcl-xFNK strongly inhibits apoptosis not by an indirect way, such as by excluding the reagent.

Then, we changed 8 amino acid residues, involved in the polar interactions among the helixes, to chemically similar residues with 7 different combinations by site-directed mutagenesis (Fig. 1B). When two amino acid residues were changed in addition to Bcl-xFNK to disturb two additional intramolecular polar interactions (Bcl-xFNDQK), the anti-apoptotic activity was decreased instead of being increased (data not shown). When two or three amino acid residues was changed with various combinations (Bcl-xAQ, Bcl-xFD, Bcl-xDQ, Bcl-xQK, Bcl-xFK, and Bcl-xDQK), no anti-apoptotic activity was enhanced (data not shown). Thus, Bcl-xFNK is the strongest anti-apoptotic factor with minimum substitutions of amino acid residues of Bcl-xL. This result indicates that the mobility or flexibility of the α5–α6 helixes of Bcl-xL should be critical to enhance the anti-apoptotic activity, suggesting that the role of the putative channel is essential for the anti-apoptotic activity, at least for enhancing the activity.

Next, Jurkat-bcl-xFNK was treated with various stimuli (Fig. 3, A–G). Bcl-xFNK made the cells more resistant than Bcl-xL against all the apoptosis-inducing stimuli as far as examined; including anti-Fas antibody (a kind of death cytokine), staurosporine (an inhibitor of protein kinases), camptothecin, hydroxyurea, trichostatin A (cell cycle inhibitors), A23187 (calcium ionophore), hydrogen peroxide, paraquat (a potent superoxide generator), serum withdrawal and heat. At day 1 after heat treatment, cell viabilities were also measured by the WST-1 assay (Fig. 3H). More dehydrogenase activity remained in Jurkat-bcl-xFNK than in Jurkatvec and Jurkat-bcl-x.

Moreover, we investigated the activity of Bcl-xFNK to suppress cell death induced by serum deprivation in CHO cells, because this cell line is frequently used in the industry to manufacture complex proteins for both therapeutic and diagnostic purposes. Two stable transfectants, CHO-bcl-x and CHO-bcl-xFNK, were confirmed to overproduce Bcl-xL or Bcl-xFNK abundantly and to the same level by Western blot analysis (Fig. 4D). The cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 without serum for 6 days (see “Materials and Methods”). CHO cells with the empty vector could form few colonies (colony formation efficiency, 2%). In their colonies, most cells were dead or dying, with large open spaces among the cells (Fig. 4C). CHO-bcl-x and CHO-bcl-xFNK cells gave
almost same colony formation efficiency. But the appearances of their colonies were distinct. In CHO bcl-x FNK colonies, only a few cells were dying and the cells firmly contacted each other without space (Fig. 4A). In CHO bcl-x colonies, more cells were dead and dying, resulting in many small or large open spaces among the cells (Fig. 4B). These results indicate that Bcl-xFNK has a much stronger activity to suppress cell death caused by serum deprivation than Bcl-xL in CHO cells.

Bcl-xFNK Conferred IL-3 Independence on the IL-3-dependent Cell Line FDC-P1—A murine promyeloid cell line, FDC-P1 requires cytokine, IL-3, for growth. Therefore, it was used to elucidate the anti-apoptotic activity of Bcl-xFNK against deprivation of the sole growth factor but not serum. We examined three independent FDC-P1 transfectants overexpressing Bcl-xFNK (FDC-P1bcl-xFNK-1 to -3) as abundantly as FDC-P1bcl-xL41 cells overexpressed Bcl-xL by Western blot analysis using the anti-Bcl-xL monoclonal antibody. The complex of the antibody with wild or mutant Bcl-xL was visualized with a fluoroimage analyzer FLA-2000 using the AttoPhos™ kit.

FIG. 3. Bcl-xFNK shows an enhanced anti-apoptotic activity in Jurkat cells against various death stimuli. A-G, parent cells (open diamond) and cells transfected with a vector (open rectangle), bcl-x (closed circle), and bcl-xFNK (open circle) were treated with anti-Fas antibody (A) at indicated concentrations; B, staurosporine (an inhibitor of protein kinase C, 20 nM); C, calcium ionophore A23187 (1 μM); D, hydrogen peroxide (0.1 mM); or E, paraquat (a potent generator of superoxide; 1 mM), or treated by serum withdrawal (F) or heat stress (G). Surviving cells were counted by trypan blue exclusion. For anti-Fas experiments, the number of cells incubated without the antibody was taken as 100% after 24 h incubation. For A23187 experiments, the average of three experiments is presented with the standard deviation (vertical bars). For the other experiments, representative results of three to four independent experiments are shown. H, an aliquot of heat-treated cells on day 1 in panel G was subjected to WST-1 assay. The activity of cells without heat treatment was taken as 100%. The average of three measurements is presented with the standard deviation (vertical bars). I, Western blot analysis. Whole cellular proteins (20 μg) prepared from Jurkat cells transfected with an empty vector (Vec), bcl-x (Bcl-x), and bcl-xFNK (FNK) were subjected to Western blot analysis using the anti-Bcl-xL monoclonal antibody. The complex of the antibody with wild or mutant Bcl-xL was visualized with a fluoroimage analyzer FLA-2000 using the AttoPhos™ kit.

FIG. 4. Bcl-xFNK inhibits death of CHO cells induced by deprivation of serum. CHO cells transfected with bcl-xFNK (A), bcl-x (B), and an empty vector (C) were plated in the medium with 3% serum. For five consecutive days, two-thirds of the medium was replaced with medium without serum. The cells were incubated in medium lacking serum from day 6. The presented photographs of colonies were taken on day 12. D, Western blot analysis. Whole cellular proteins (20 μg) prepared form CHO parent (Par) cells and CHO cells transfected with an empty vector (Vec), bcl-x (Bcl-x), and bcl-xFNK (FNK) were subjected to Western blot analysis using the anti-Bcl-xL monoclonal antibody. The complex of the antibody with wild or mutant Bcl-xL was visualized with a fluoroimage analyzer FLA-2000 using the AttoPhos™ kit.
Hydrogen Bonds in Bcl-xL Regulate Anti-apoptotic Activity

Bcl-xL is more suitable to design an anti-apoptotic factor by remodeling its structure because of its own stronger anti-apoptotic activity and of its refined structure determined by the x-ray crystallography. We focused on the role of the a5-a6 helices of Bcl-xL and made them more mobile to be inserted into a membrane to generate an ion channel. Actually, we have constructed the super anti-apoptotic factor Bcl-xFNK by introducing three amino acid substitutions Y225E, Q260N and R165K in rat Bcl-xL. Bcl-xFNK suppressed cell death of Jurkat against a wide variety of stimuli more strongly than Bcl-xL. Disruption of three intramolecular hydrogen bond interactions generated a novel mutant, Bcl-xFNK with a gain-of-function phenotype.

Staurosporine (13), camptothecin (14, 15), trichotestatin A (16), anti-Fas (17, 18), calcium ionophore A23187 (19), hydrogen peroxide (20, 21), and heat stress (22) finally activate the caspase cascade to execute cell death. The function(s) of Bcl-xL at the molecular level is still unknown, although it has been proposed that Bcl-xL mainly locates at mitochondria membranes, interacts with a complex of Apaf-1 and pro-caspase-9 as well as anti-apoptotic factor Bax (23), suppresses release of cytochrome c from mitochondria (24), regulates mitochondrial membrane potential (25), interacts with VDAC (voltage-dependent anion channel) to inhibit the permeability transition pore from opening (26, 27), and forms ion channels (2, 3). Anti-apoptotic Bcl-xL, Bcl-x2L and Bcl-2 and pro-apoptotic Bax can form ion channels in vitro. A putative pore-forming domain is hypothetically assigned to the a5-a6 region of these proteins. Bcl-2 lacking the putative a5-a6 region failed to form ion channels in synthetic membranes in vitro (4) and the putative a5-a6 region was essential for Bcl-2 in promoting cell survival (5). The central a5-a6 region in the Bcl-2 family proteins apparently plays an important and distinct role in apoptosis regulation. It is stressed that the ease with which the a5-a6 region can insert into synthetic membranes appears to enhance the function of Bax and Bcl-xL (6). This could be a common step for some of the Bcl-2 family proteins to express their activity. It is important and necessary in further studies to investigate whether the ion channel activity of Bcl-xFNK is enhanced in vitro.

To elucidate the role of the putative pore-forming domain of Bcl-xL, Bcl-xFNK would provide a possibility of importance for generating the channel to express the anti-apoptotic activity.

**DISCUSSION**

**Bcl-xFNK transfectants of FDC-P1 and Jurkat cells—**Cytoplasmic lysates were prepared from the FDC-P1 transfectants cultured in the presence of IL-3 (Fig. 5C). All bcl-xFNK transfectants tested had a level of MAP kinase activity 3 times higher than that of the vector transfectant. There was no difference among the MAP kinase activities of two independent bcl-x transfectants (clones 41 and 124, both overexpressing Bcl-xL to the same extent [data not shown]), the parent FDC-P1 cell and the vector transfectant. We have obtained the same results using lysates of the cells cultured without IL-3 for 1–3 days (data not shown). We also prepared cytoplasmic lysates from transfectants of Jurkat, BaF/3, and CHO cells. In Jurkat bcl-xFNK cells, the MAP kinase was activated twice more than in Jurkat bcl-x, whereas the MAP kinase activity of Jurkat bcl-x was almost the same or somewhat lower than that of the parent cells and of Jurkat vec cells (Fig. 5C). In CHO bcl-xFNK cells and BaF/3 bcl-xFNK cells, there was no significant activation of the MAP kinase, compared within their parent cells and their bcl-x transfectants. These results indicate that Bcl-xFNK enhances the p42/p44 MAP kinase activity in a cell-type specific manner. It seems likely that the IL-3-independent growth of FDC-P1 cells overexpressing Bcl-xFNK is due to enhanced p42/p44 MAP kinase activity, because IL-3 impinges on cells to activate the p42/p44 MAP kinase (12).

**FIG. 5.** Bcl-xFNK confers IL-3 independent growth on FDC-P1 cells and activates the p42/p44 MAP kinase activity in FDC-P1 and Jurkat cells. A, Western blot analysis. Whole cellular proteins (20 μg) prepared from FDC-P1 cells transfected with an empty vector (Vec), bcl-x (Bcl-x), bcl-xFNK (FNK clones 1 to 3), bcl-xQK (QK), and bcl-xFK (FK) were subjected to Western blot analysis using the anti-Bcl-xL monoclonal antibody. The complex of the antibody with wild or mutant Bcl-xL was visualized by the chemiluminescence method using the RENNAISSANCE™ kit or with a fluorography analyzer FLA-2000 using the AttoPhos™ kit. B, IL-3 independent growth of FDC-P1 bcl-xFNK cells. Three independent clones of FDC-P1 bcl-xFNK (FNK clones 1 to 3), FDC-P1 bcl-xQK (QK), FDC-P1 bcl-xFK (FK), and FDC-P1 vec (Vector) were washed and incubated in medium containing serum but lacking IL-3. The surviving cells were counted by trypan blue exclusion. The number of cells on day 0 was taken as 100% and the vertical axis is presented in a logarithmic scale. Representative results of three independent experiments are shown. C, the p42/p44 MAP kinase activity. Cytoplasmic lysates were prepared from FDC-P1 and Jurkat parent cells and their transfectants containing vector (V), bcl-x (Bcl-x), and bcl-xFNK (FNK). Activities of the p42/p44 MAP kinase in the lysates were measured with the BIORAD p42/p44 MAP kinase enzyme assay system (Amersham Pharmacia Biotech) and normalized against protein amount. Normalized MAP kinase activity of FDC-P1 vec or Jurkat vec was taken as 1 in FDC-P1 experiments or Jurkat experiments, respectively. The average of three to four independent preparations is presented with the standard deviation (vertical bars).
In this study, we showed that the other combinations of amino acid mutation did not enhance the apoptosis suppressing activity, but that only the combination of the three amino acid substitutions (Y22F, Q26N, and R165K) did. This finding indicates that the substitutions of the amino acids themselves are not important, but that the mobility or flexibility of the δ5-δ6 region of Bcl-xL is critical to enhancing the anti-apoptotic activity.

In addition to the enhanced function, a novel feature of Bcl-xFNK appeared in FDC-P1 cells, that is, IL-3-independent growth. Overproduction of Bcl-2 or Bcl-xL results in a prolonging of cell survival against death stimuli but never leads to proliferation. Cooperation of Bcl-2 and c-Myc conferred IL-3 independence upon the growth factor-dependent pre-B cells (28). It is unlikely that Bcl-xFNK gains the ability to regulate gene expression like transcription factor c-Myc. In all FDC-P1 cells overexpressing Bcl-xFNK as well as Jurkat bcl-xL, the p42/p44 MAP kinase activity was shown to be enhanced. IL-3 has been reported to activate the MAP kinase (12). It is speculated that Bcl-xFNK would directly or indirectly activate the MAP kinase. It is stressed that Bcl-xFNK allowed IL-3 independent growth in a cell type-specific manner. Bcl-xFNK may indirectly activate the MAP kinase through interaction(s) with factor(s) which are present in FDC-P1 but absent in Ba/F3.

Bag-1 (Bcl-2-associated athanogene 1) interacts with many proteins including Bcl-2 and suppresses apoptosis (29). Overexpression of Bag-1 confers IL-3 independence upon Ba/F3 cells (30). We could not detect any Bcl-x or Bcl-xFNK co-immunoprecipitated with the anti-Bag-1 antibody (C-16; Santa Cruz Biotech), from FDC-P1 overexpressing Bcl-xL or Bcl-xFNK, respectively, regardless of the presence of IL-3 (data not shown). Expression levels of Bag-1 were the same in these cells (data not shown). It is not likely that Bag-1 is involved in the mechanism by which FDC-P1 cells gain IL-3 independence through Bcl-xFNK.

Nutrient limitation, oxygenation (oxygen stress), and mechanical agitation (hydrodynamic stress) are very important factors in cell death in the bioreactor environment. Bcl-2 has been reported to increase maximum viable cell density and mechanical agitation (hydrodynamic stress) are very important factors in cell death in the bioreactor environment. Bcl-2 has been shown to increase maximum viable cell density and viability of CHO cells in batch culture and to maintain viability of thymidine-treated CHO cells (31). It has recently been shown that Bcl-xL consistently affords CHO cells a high degree of protection from various culture insults, compared with Bcl-2 (32). However, the anti-apoptotic activities are not strong enough to protect the cultured cells against apoptosis in industrial uses. Thus, Bcl-xFNK may not be extremely useful for making commercially important animal cell lines robust to culture insults.

In terms of clinical applications of the future, Bcl-xFNK may more effectively protect neuronal cells from degeneration or cell death caused by brain injury and ischemia than Bcl-2 and Bcl-xL. In fact, we have shown that ectopic expression of Bcl-2 or Bcl-xL delays neuronal degeneration in the hippocampus of adult rat brain (33). In addition, Bcl-xFNK may play an important role to cure sick cells due to collapse of Ca\(^{2+}\) homeostasis, because exposure of cells to calcium ionophore revealed the relative robustness of the cells overexpressing Bcl-xFNK.

Finally, we emphasize that this construction is not a simple remodeling of the Bcl-xL protein by random selection, but was designed based on the idea that the insertion of the putative pore-forming domain into membranes is essential for expressing the anti-apoptotic activity. Further analysis of Bcl-xFNK with a gain-of-function phenotype could provide new insight into the function of Bcl-xL in apoptosis regulation.

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