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Bcl-x_L regulates apoptosis by heterodimerization-dependent and -independent mechanisms

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A hydrophobic cleft formed by the BH1, BH2 and BH3 domains of Bcl-x_L is responsible for interactions between Bcl-x_L and BH3-containing death agonists. Mutants were constructed which did not bind to Bax but retained anti-apoptotic activity. Since Bcl-x_L can form an ion channel in synthetic lipid membranes, the possibility that this property has a role in heterodimerization-independent cell survival was tested by replacing amino acids within the predicted channel-forming domain with the corresponding amino acids from Bax. The resulting chimera showed a reduced ability to adopt an open conductance state over a wide range of membrane potentials. Although this construct retained the ability to heterodimerize with Bax and to inhibit apoptosis, when a mutation was introduced that rendered the chimera incapable of heterodimerization, the resulting protein failed to prevent both apoptosis in mammalian cells and Bax-mediated growth defect in yeast. Similar to mammalian cells undergoing apoptosis, yeast cells expressing Bax exhibited changes in mitochondrial properties that were inhibited by Bcl-x_L through heterodimerization-dependent and -independent mechanisms. These data suggest that Bcl-x_L regulates cell survival by at least two distinct mechanisms; one is associated with heterodimerization and the other with the ability to form a sustained ion channel.

Keywords: apoptosis/Bax/Bcl-x_L/ion channel/yeast

Introduction

Although many proteins are known to be involved in the control of apoptosis, members of the evolutionarily conserved Bcl-2 family are thought to be central regulators (Kroemer, 1997; Chao and Korsmeyer, 1998). Recent data suggest that these proteins function at or near a point in the cell death pathway that dictates whether or not cells are committed to die. Progression past this point initiates an execution and degradation phase whereby downstream caspases, which are apoptosis-associated cysteine proteases, become activated and catalyze the morphological and biochemical changes associated with apoptosis. These changes include cell volume shrinkage, cytoskeletal reorganization, DNA condensation and DNA fragmentation. Although the Bcl-2 family members have been found to play a critical role in the regulation of apoptosis, the exact biochemical function(s) that is utilized by these proteins to regulate cell survival is unclear.

Members of the Bcl-2 family can be generally divided into proteins that either promote or inhibit apoptosis (Chao and Korsmeyer, 1998). Members of the former include Bax and Bak, while members of the latter include Bcl-2 and Bcl-x_L. Although it has been suggested that heterodimerization between death agonists and antagonists regulates their respective functions, the importance of heterodimerization remains unresolved. For example, some mutants of Bcl-2 and Bcl-x_L that fail to bind to Bax also fail to protect against apoptosis (Yin *et al*., 1994; Sedlak *et al*., 1995). However, other mutants of Bcl-x_L that fail to bind to Bax retain significant anti-apoptotic function (Cheng *et al*., 1996). One possibility is that other dimerization partners are involved in the ability of Bcl-x_L to regulate cell survival. Anti-apoptotic Bcl-2 family members have been shown to interact with various other death agonists of the Bcl-2 family and with non-Bcl-2 family proteins, including Raf-1 (Wang *et al*., 1996), calcineurin (Shibasaki *et al*., 1997), CED-4 (Chinnaiyan *et al*., 1997b; James *et al*., 1997; Spector *et al*., 1997; Wu *et al*., 1997b), Apaf-1 (Hu *et al*., 1998; Pan *et al*., 1998) and caspases (Clem *et al*., 1998). This extensive network of protein–protein interactions between Bcl-2 family members and non-Bcl-2 family members makes the relative contribution of each of these interactions to the regulation of apoptosis difficult to address. However, the interaction with the *Caenorhabditis elegans* CED-4 protein and its mammalian homolog Apaf-1 has been interpreted as evidence for one major mechanism by which Bcl-x_L prevents apoptosis (Hu *et al*., 1998; Pan *et al*., 1998). Since CED-4 and Apaf-1 are involved in the processing and activation of downstream caspases that carry out the degradation phase of apoptosis (Chinnaiyan *et al*., 1997a; Seshagiri and Miller, 1997; Wu *et al*., 1997a; Zou *et al*., 1997), Bcl-x_L may prevent cell death in mammalian cells by sequestering Apaf-1.

In addition to regulating cell survival by sequestering other proteins, Bcl-2 and Bcl-x_L may possess an intrinsic biochemical activity to control apoptosis without binding to death agonists. Consistent with their structural homology to the bacterial pore-forming domains, Bcl-x_L, Bcl-2 and Bax have been shown to form ion channels in biological membranes (Antonsson *et al*., 1997; Minn *et al*., 1997; Schendel *et al*., 1997; Schlesinger *et al*., 1997). The ion channels formed by these proteins are large conductance

channels that are either weakly anion or cation selective, with differences in channel properties existing between death agonists and antagonists. These proteins can reside on various subcellular membranes, including the outer mitochondrial membrane. Recent data suggest that cells undergoing apoptosis exhibit mitochondrial swelling and alterations in the mitochondrial membrane potential that lead to the release of pro-apoptotic mitochondrial proteins, such as cytochrome *c* (Susin *et al*., 1996; Vander Heiden *et al*., 1997). Cytochrome *c* is a necessary cofactor for Apaf-1 to activate downstream caspases (Zou *et al*., 1997). Bcl-2 and Bcl-x_L have been shown to inhibit these apoptosis-related changes in mitochondria and to inhibit the release of cytochrome *c* (Kluck *et al*., 1997; Vander Heiden *et al*., 1997; Yang *et al*., 1997). Furthermore, Bcl-2 has recently been shown to enhance the efflux of protons from the mitochondria (Shimizu *et al*., 1998). This has led to the suggestion that the ion channel activity of Bcl-2 family members can control apoptosis by influencing the permeability of the subcellular membranes to which it distributes.

The purpose of this study was to establish the functional importance of the ability of Bcl-x_L to heterodimerize with BH3-containing death agonists in relation to its ability to regulate apoptosis. Furthermore, the mechanism(s) by which Bcl-x_L might control cell survival independently of heterodimerization were also investigated. Structural information that provides the critical determinants involved in the interaction between Bcl-x_L and BH3-containing death agonists was used to design mutants that disrupt Bcl-x_L heterodimerization. These mutants were found to retain significant anti-apoptotic function in the absence of heterodimerization. Further structure–function studies were performed to identify the region of the protein responsible for the heterodimerization-independent regulation of cell survival. Mutations within residues surrounding the two central hydrophobic helices that are predicted to be involved in ion channel formation altered ion channel properties and disrupted heterodimerization-independent cell survival. These data suggest that both ion channel function and heterodimerization modulate the ability of Bcl-x_L to control apoptosis.

Results

Bcl-x_L can regulate apoptosis independently of heterodimerization

We have demonstrated previously that the BH1, BH2 and BH3 domains of Bcl-x_L form a hydrophobic cleft on the

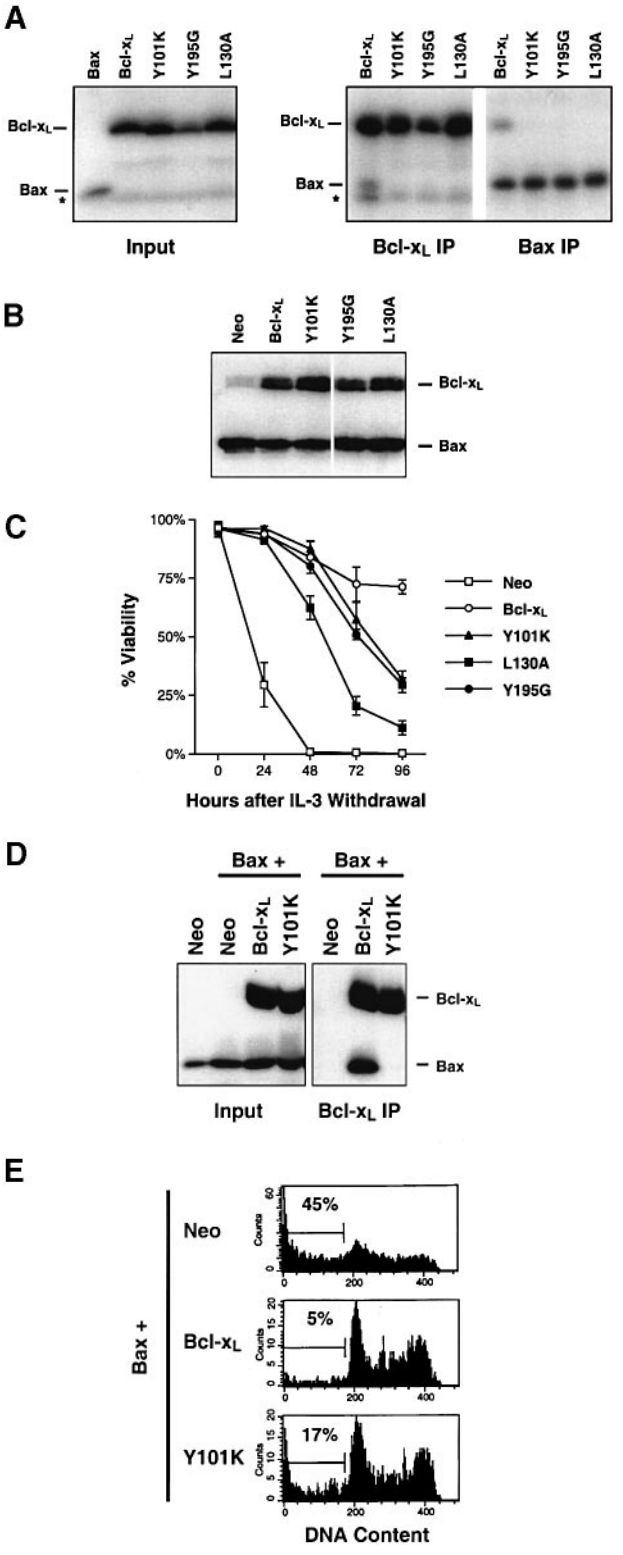

Fig. 1. BH1, BH2, and BH3 mutants of Bcl-x_L that disrupt heterodimerization with Bax retain anti-apoptotic function. (**A**) Bcl-x_L, Bcl-x_L Y101K, Bcl-x_L L130A or Bcl-x_L Y195G were *in vitro*-translated and incubated with *in vitro*-translated Bax. Heterodimerization was determined by immunoprecipitation with either an anti-Bcl-x antibody or an anti-Bax antibody. The input is shown. The asterisk represents a premature termination product of the Bcl-x_L *in vitro* translation. (**B**) Bcl-x_L, Bcl-x_L Y101K, Bcl-x_L L130A, Bcl-x_L Y195G or an empty expression vector (Neo) was stably transfected into FL5.12 cells. Shown is an immunoblot with a cocktail of rabbit polyclonal antibodies to Bcl-x and Bax. (**C**) Stable transfectants of the indicated FL5.12 cells were deprived of IL-3 and viability was measured over the course of 96 h by propidium iodide exclusion. Shown are means ± standard deviations from three independent experiments. (**D**) Cell lysates from 293 cells that were transiently transfected with Bcl-x_L, Bcl-x_L Y101K or an empty expression plasmid (Neo) were immunoprecipitated with an anti-Bcl-x antibody and examined for association with co-transfected Bax. Immunoprecipitated products and the input (25%) were visualized by immunoblotting with a cocktail of rabbit polyclonal antibodies to Bcl-x and Bax. (**E**) Bax and a GFP-expression plasmid was transiently transfected with either Bcl-x_L, Bcl-x_L Y101K or an empty expression plasmid (Neo) into 293 cells. At 24 h post-transfection, both suspension and adherent cells were harvested, fixed and stained with propidium iodide. The amount of apoptosis was measured by gating on the GFP-expressing cells and by quantitating the amount of DNA hypodiploidy using flow cytometry. At least 1000 events were analyzed. The amount of DNA hypodiploidy is indicated.

surface of the molecule (Muchmore *et al*., 1996). This hydrophobic cleft provides a binding site for the BH3 domain of various pro-apoptotic Bcl-2 family members, such as Bax and Bak, and is critical for heterodimerization between Bcl-x_L and these pro-apoptotic proteins (Sattler *et al*., 1997). Various amino acid residues from the BH1, BH2 and BH3 domains of Bcl-x_L are involved in forming hydrophobic interactions with the complexed BH3 domain. These critical residues include Y101 from BH3, L130 from BH1, and Y195 from BH2. Previously reported mutants of Bcl-x_L that fail to bind to Bax were mutated at residues that are not predicted to be involved in direct interaction with the Bax BH3 (Sedlak *et al*., 1995; Cheng *et al*., 1996). Therefore, in order to minimize possible secondary effects, mutants of Bcl-x_L were constructed that either substituted Y101 with K, L130 with A, or Y195 with G. Although these mutations disrupt residues that are directly involved in forming hydrophobic interactions with the BH3 domain of death agonists, they are not involved in intramolecular interactions as defined by the Bcl-x_L structure. Each of these mutants was first tested for the ability to heterodimerize with Bax by *in vitro* co-immunoprecipitation studies. As seen in Figure 1A, co-immunoprecipitation with either an anti-Bcl-x_L antibody or an anti-Bax antibody failed to show any interaction between the Bcl-x_L mutants and Bax. These mutants were then stably transfected into the IL-3-dependent cell line FL5.12 and analyzed for the ability to protect against apoptosis. As seen in Figure 1B, each of the BH1, BH2 and BH3 mutants was expressed at equivalent levels to wild-type Bcl-x_L. Consistent with the *in vitro* interaction studies, these mutants failed to bind to Bax in FL5.12 cells (see Figure 4A and data not shown). Despite the inability to heterodimerize with Bax, each mutant still conferred significant protection from apoptosis induced by IL-3 deprivation (Figure 1C). This ability to prevent apoptosis did not correlate with binding to Apaf-1. Neither wild-type Bcl-x_L nor the Bcl-x_L mutants displayed significant binding to Apaf-1 under the same conditions used to examine heterodimerization with Bax (data not shown).

The ability of non-binding mutants to protect 293 cells against apoptosis induced by Bax expression was also tested. Bax and either Bcl-x_L or Bcl-x_L Y101K were transiently transfected into 293 cells. Protein interactions were studied by co-immunoprecipitation and cell death was quantitated by including a green fluorescent protein (GFP)-expression plasmid in the transfection and measuring the amount of DNA hypodiploidy within the GFP-expressing cells at 24 h post-transfection. As shown in Figure 1D and E, despite the inability to heterodimerize with Bax, Bcl-x_L Y101K retained significant anti-apoptotic activity against Bax-induced cell death. Similar results were also obtained with the non-binding Bcl-x_L L130A and Bcl-x_L Y195G (data not shown). Thus, although heterodimerization contributes to the anti-apoptotic properties of Bcl-x_L, it may not be the sole factor since the protein retains significant anti-apoptotic properties in the absence of binding to Bax.

Bcl-x_L exhibits heterodimerization-independent inhibition of Bax-mediated growth defect in yeast

Although Bcl-x_L Y101K also did not interact with other BH3-containing death agonists such as Bak and Bad

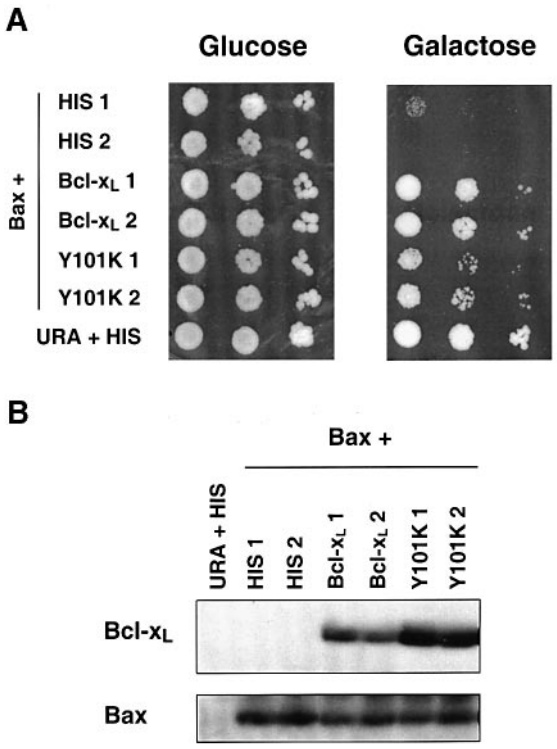

Fig. 2. Bcl-x_L can inhibit Bax-mediated toxicity in yeast in a manner that is independent of heterodimerization. The yeast *S.cerevisiae* was transformed with a multi-copy expression plasmid containing the uracil selection marker and expressing human Bax under the control of a galactose-inducible promoter. Several clones were selected and found to be growth inhibited when Bax expression was induced with galactose. A representative clone was transformed again with a multi-copy expression plasmid containing the histidine selection marker and expressing either human Bcl-x_L or Bcl-x_L Y101K under the control of a galactose-inducible promoter. Several subclones of the double-transformants were selected and characterized, but shown here are representative subclones. (**A**) The ability of Bcl-x_L and Bcl-x_L Y101K to suppress Bax-mediated toxicity was determined by growing yeast cells overnight in galactose media and spot-testing on galactose plates starting with an equal number of yeast cells as determined by taking the OD_{600}. Ten-fold serial dilutions were used in the spot-test. Plates were grown at 30°C for 2–3 days. (**B**) An immunoblot for Bcl-x_L and Bax to demonstrate protein expression levels after 18 h in galactose media.

(data not shown), the existence of multiple Bcl-2 family members in mammalian cells prevents us from eliminating the possibility that the mutants of Bcl-x_L that fail to interact in our assays are still regulating cell survival in a heterodimerization-dependent manner. In addition, Bcl-2 and/or Bcl-x_L have been reported to interact with many other proteins that are not Bcl-2 family members. To address these concerns, we took advantage of the finding that Bax expression is toxic to the yeast *Saccharomyces cerevisiae* (Zha *et al*., 1996). Bax was placed under the control of a galactose-inducible promoter and transformed into yeast. As shown by growth on galactose media, yeast that were induced to express Bax demonstrated a severe growth defect but grew normally when Bax expression was repressed by glucose (Figure 2A). In order to keep Bax levels as constant as possible, a representative Bax-transformant was transformed again with Bcl-x_L, Bcl-x_L Y101K or an empty expression vector (Figure 2B). Bcl-x_L was able to suppress the Bax-mediated growth defect, as was the non-binding Bcl-x_L Y101K. As in mammalian cells, Bcl-x_L Y101K did not protect as well as wild-type

Bcl-x_L, suggesting that heterodimerization does contribute to the protective properties of Bcl-x_L. Yeast expressing Bcl-x_L or Bcl-x_L Y101K alone grew normally (data not shown). Thus, results from both mammalian and yeast cells suggest that Bcl-x_L is able to protect against apoptosis and/or Bax in a manner that is independent of heterodimerization. These results also suggest that Bax and Bcl-x_L are able to function in the absence of other components of the metazoan apoptotic machinery, perhaps through an intrinsic activity that is important in regulating cell survival.

The heterodimerization-independent regulation of cell survival by Bcl-x_L relies on an amino acid region surrounding helix 5 and helix 6

Bcl-x_L, Bcl-2 and Bax each form ion channels in biological membranes, and this biochemical function has been proposed to be involved in the ability of these proteins to regulate cell survival. Studies investigating the channel-forming properties of the structurally related diphtheria toxin pore-forming domain have shown that the two central core helices, which are equivalent to helix 5 and helix 6 in the Bcl-x_L structure, are sufficient to recapitulate the channel properties of the entire pore-forming domain (Silverman *et al*., 1994b). In addition, charge reversal mutations in the region surrounding the hairpin that connect the two central helices alter the channel properties and prevent the full-length toxin from killing mammalian cells (Silverman *et al*., 1994a). Therefore, we inspected the region surrounding the hairpin that connects helix 5 and helix 6 in Bcl-x_L. Similar to diphtheria toxin, there are several charged residues in the 13 amino acid region from E153 to R165 (Figure 3A and B). Comparison of this region between multiple Bcl-2 family members revealed that this region bears a net negative charge in several anti-apoptotic proteins and a net positive charge in the pro-apoptotic proteins Bax and Bak. To test the contribution of the charge character in this region to the cell survival properties of these proteins, the region surrounding the Bcl-x_L helix 5 and helix 6 hairpin was replaced with the corresponding region from Bax. This chimera is referred to as XB.

The XB mutation is not predicted to disrupt the BH3-interacting hydrophobic cleft of Bcl-x_L since the region replaced in XB is relatively distant from the hydrophobic cleft in the three-dimensional structure of the protein (Figure 3B and C). This was confirmed by co-immunoprecipitation studies of FL5.12 cells stably transfected with XB. As shown in Figure 4A, XB retained the ability to heterodimerize with endogenously expressed Bax at levels that were comparable to wild-type Bcl-x_L. Since the hydrophobic cleft that is involved in complex formation requires the juxtaposition of the BH1, BH2 and BH3 domains, the ability of XB to heterodimerize with Bax suggests that the mutant is properly folded. As shown in Figure 4B, the ability to complex with Bax correlated with the ability of XB to protect as well as the wild-type protein against apoptosis induced by growth factor deprivation.

Since mutants of Bcl-x_L that fail to bind to Bax can still prevent apoptosis, we sought to determine whether the heterodimerization-independent mechanism by which Bcl-x_L prevents cell death is influenced by the XB mutation. To test this, Y101 was replaced with K in XB, and the resulting double mutant was stably transfected into FL5.12 cells. As shown in Figure 4A, the introduction of the Y101K mutation to wild-type Bcl-x_L or XB resulted in the loss of Bax-binding. Although Bcl-x_L Y101K retained a majority of its anti-apoptotic properties in response to growth factor withdrawal, when the XB and Y101K mutations were combined, this resulted in the near complete loss of anti-apoptotic function (Figure 4B). This was not due to protein instability since XB/Y101K was comparably stable to wild-type Bcl-x_L, Bcl-x_L Y10K and XB as determined by protein half-life experiments using cycloheximide (data not shown).

The failure of XB/Y101K to counter cell death was confirmed in mammalian 293 cells induced to undergo apoptosis by Bax. As shown in Figure 4C, co-transfection of Bax with Bcl-x_L or XB resulted in complete protection, and co-transfection with Bcl-x_L Y101K resulted in partial protection. In contrast, XB/Y101K failed to protect against Bax-mediated apoptosis. This failure was not due to a lack of protein expression since Bcl-x_L and all mutants of Bcl-x_L were highly expressed as measured by flow cytometry of intracellularly stained cells (Figure 4D). In addition, co-immunoprecipitation studies confirmed that in 293 cells, Bcl-x_L and XB were able to complex with Bax, while Bcl-x_L Y101K and XB/Y101K failed to interact with Bax (data not shown). Transfection of either XB or XB/Y101K by themselves had no effect (data not shown). Thus, Bcl-x_L appears to protect mammalian cells against multiple apoptotic stimuli by at least two distinct mechanisms. One mechanism involves heterodimer formation with Bax and presumably other BH3-containing death agonists, while the other mechanism requires a property that is altered by the XB mutation.

To determine whether the XB/Y101K double mutant was also defective in protecting yeast cells from Bax, the same Bax-expressing yeast clone presented in Figure 2 was transformed with Bcl-x_L, the various Bcl-x_L mutants or a control plasmid. The double-transformants were tested for growth on galactose-containing media. As shown in Figure 5A, yeast cells that express Bax demonstrated a severe growth-defect that is suppressed by co-expression of either Bcl-x_L or XB, and partially suppressed by Bcl-x_L Y101K. In contrast, XB/Y101K failed to restore growth to yeast expressing Bax, despite being expressed at levels comparable to Bcl-x_L and other Bcl-x_L mutants (Figure 5B). Bax was also induced to comparable levels in all transformants (data not shown). The few large colonies that appear may represent spontaneous mutants. Neither XB nor XB/Y101K had a noticeable phenotype when expressed alone (data not shown). Thus, even in yeast, the heterodimerization-independent mechanism that protects against Bax is altered by the XB mutation.

The disruption of the heterodimerization-independent regulation of cell survival correlates with alterations in Bcl-x_L ion channel properties

In order to determine whether XB and XB/Y101K exhibit alterations in Bcl-x_L ion channel properties, these proteins, along with wild-type Bcl-x_L and Bcl-x_L Y101K, were purified in recombinant form and tested in a planar lipid bilayer system. Analytical SDS–PAGE and NMR confirmed the identity, purity and proper folding of each

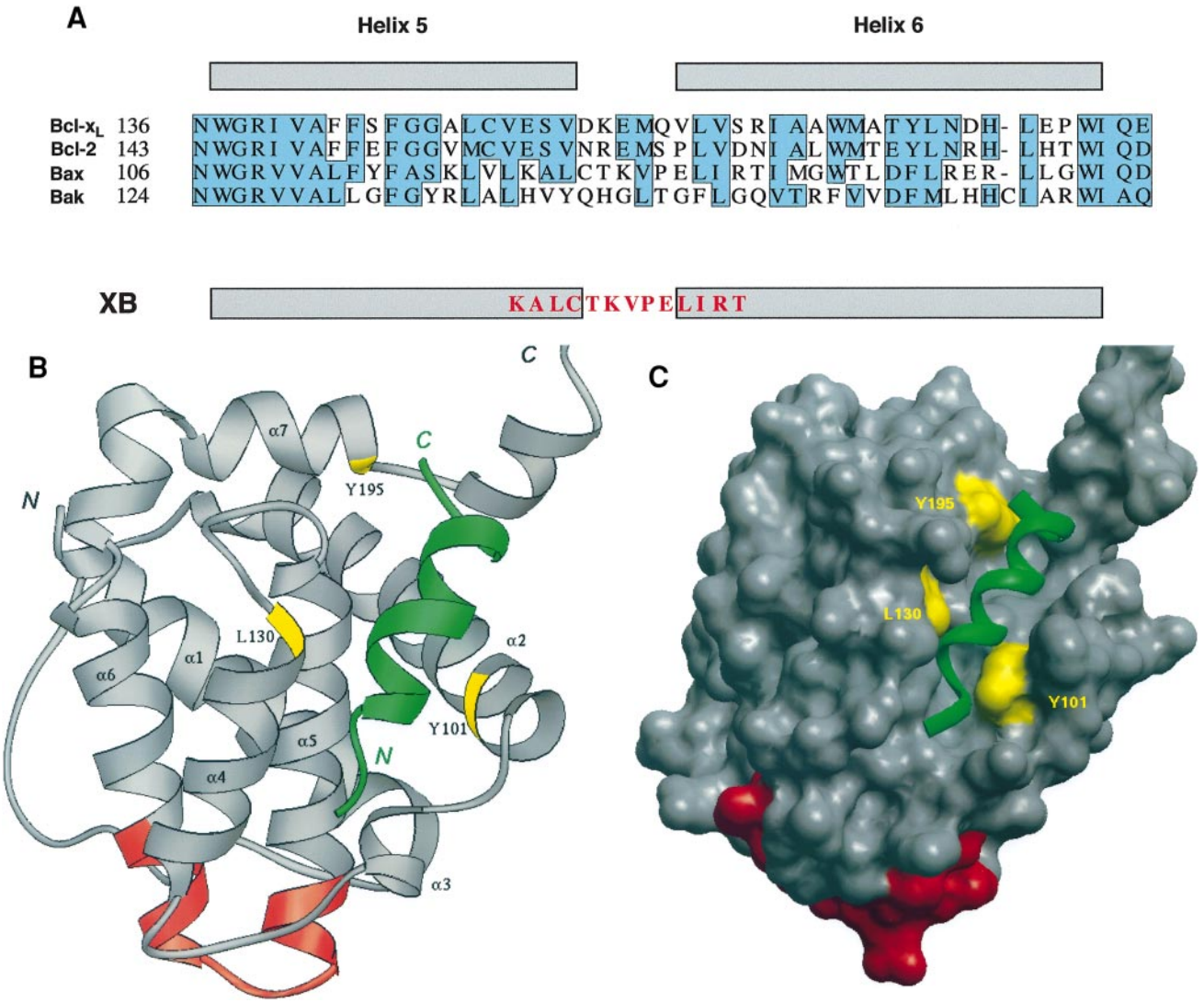

Fig. 3. Design and illustration of the XB mutants. (**A**) Comparison of the amino acid sequence surrounding helix 5 and helix 6 of various Bcl-2 family members (similar amino acids are outlined in blue). The amino acids comprising the central helix 5 and helix 6 from Bcl-x_L (represented by the outline filled in gray) and the predicted amino acids comprising these helices in other Bcl-2 family members is indicated. The 13 amino acids of the XB mutants that correspond to the amino acids from Bax are shown in red. This 13 amino acid region has a net negative charge in Bcl-2 and Bcl-x_L. In Bax and Bak this region has a net positive charge. (**B**) Ribbon representation and (**C**) surface representation of the Bcl-x_L/BH3 peptide complex (Sattler *et al*., 1997), and the locations of the Y101K, L130A, Y195G, and XB mutations. Bcl-x_L is shown in gray. Residues Y101, L130, and Y195 of Bcl-x_L are shown in yellow, and the region that is converted to the corresponding residues from Bax in the XB mutants is shown in red.

of the recombinant proteins (data not shown). Current fluctuations in response to a continuous voltage ramp from –80 to 20 mV were measured after addition of wild-type Bcl-x_L to a formed planar lipid bilayer in an asymmetric 150:15 mM (*cis*:*trans*) KCl gradient at pH 7.2. As shown in Figure 6A, the Bcl-x_L-associated increase in membrane conductance resulted in current fluctuations that behave in nearly an Ohmic fashion, i.e. there is a linear relationship between voltage and current. This channel had a reversal potential of approximately –30 mV, indicating that the permeation pathway is cation selective (P_K/P_{Cl} = 4.31). Furthermore, as previously reported for Bcl-2 (Schlesinger *et al*., 1997), the Bcl-x_L ion channel resided mostly in an open conductance state, although some channels that are predominantly in a closed state can be seen infrequently (Minn *et al*., 1997). The predominantly open channels were characterized by brief, infrequent closures to the zero current level. As shown in Figure 7A, channel recordings at both positive (20 mV) and negative (–60 mV) holding potentials also revealed a mostly open channel. At both 20 and –60 mV, the main conductance state was ~550 pS. Similar results were obtained with Bcl-x_L Y101K (data not shown).

The XB mutants were also tested in a planar lipid bilayer under the same conditions to determine whether the replacement of the 13 amino acids that surround helix 5 and helix 6 in XB and XB/Y101K resulted in altered ion channel properties as compared to wild-type Bcl-x_L and Bcl-x_L Y101K. Both XB and XB/Y101K gave similar results. As shown by the channel activity in response to a –80 to 20 mV voltage ramp in Figure 6B, the most frequently observed channel formed by the XB mutants was not Ohmic over the entire voltage range. Current fluctuations to the open state were favored at

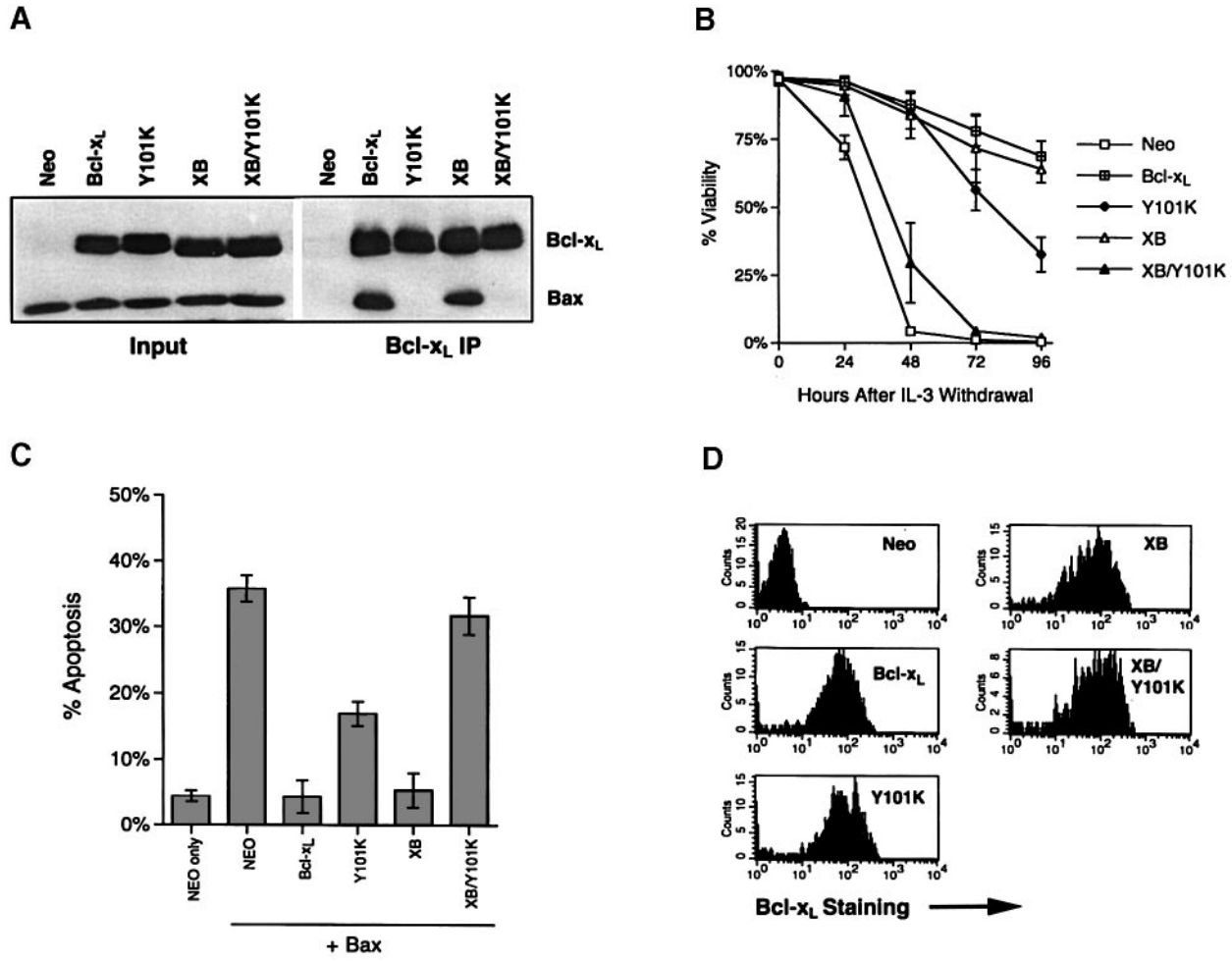

Fig. 4. The XB mutation disrupts the heterodimerization-independent anti-apoptotic function of Bcl-x_L. (**A**) FL5.12 cells were stably transfected with Bcl-x_L, the indicated Bcl-x_L mutants or an empty expression plasmid as a control (Neo). Bulk transfectants were lysed and immunoprecipitated with an anti-Bcl-x monoclonal antibody. The input (25%) and the immunoprecipitated products were resolved by SDS–PAGE and immunoblotted using a cocktail of rabbit polyclonal antibodies against Bcl-x and Bax. (**B**) Bulk transfectants of the indicated FL5.12 cells were deprived of IL-3 and viability was measured over the course of 96 h by propidium iodide exclusion. Shown are means ± standard deviations from three independent experiments. (**C**) Bax was co-transfected with Bcl-x_L, Bcl-x_L mutants or an empty expression plasmid (Neo), along with a GFP-expression plasmid into 293 cells. At 24 h post-transfection, both suspension and adherent cells were harvested, fixed and stained with propidium iodide. The amount of apoptosis was measured by gating on the GFP-expressing cells and quantitating the amount of DNA hypodiploidy using flow cytometry. Shown are means ± standard deviations from three independent experiments. (**D**) Bcl-x_L, mutants of Bcl-x_L or an empty expression plasmid were transiently transfected along with a GFP-expression plasmid into 293 cells. At 24 h post-transfection, cells were harvested, fixed and intracellularly stained with an anti-Bcl-x monoclonal antibody, followed by a PE-conjugated secondary antibody. Flow cytometry was used to quantitate the amount of Bcl-x_L or mutant Bcl-x_L protein expression by gating on GFP-expressing cells and measuring PE fluorescence.

positive electrical potentials. Furthermore, compared with the ion channels formed by wild-type Bcl-x_L or Bcl-x_L Y101K, the XB mutant channels frequently resided at lower conductance states that were at or near the closed state. As shown in Figure 7B, XB mutant channels were characterized by openings to lower conductance states compared with the Bcl-x_L channel. For example, at a membrane potential of 20 mV the 550 pS conductance state, which is the major conductance state of the Bcl-x_L channel, occurred relatively infrequently with the XB mutant channel. Instead, the XB mutant channel primarily opened to a conductance state of ~250 pS or less. At a membrane potential of –60 mV the closed state predominated.

Although the most commonly observed ion channels formed by the XB mutants were primarily inactive, occasionally channels formed by the XB mutants were mainly in an open state at positive electrical membrane potentials (Figures 6C and 7C). However, this predominantly open variant of the XB mutant channel either closed or transitioned to lower conductance states in response to negative electrical potentials. As shown by the sample channel records in Figure 7C, at a membrane potential of 20 mV the predominant conductance state was ~550 pS, similar to wild-type Bcl-x_L. However, at a membrane potential of –60 mV, the channel frequently resided at lower conductance states of ~200 and ~100 pS. The reversal potentials of the channels formed by the XB mutants were approximately –30 mV under all conditions (Figure 6), indicating that the XB mutation does not alter K^+ selectivity.

Bcl-x_L influences mitochondrial properties through heterodimerization-dependent and -independent mechanisms

Our data suggest that the ion channel activity of Bcl-x_L may be involved in protecting against growth factor deprivation-induced apoptosis in FL5.12 cells, Bax-

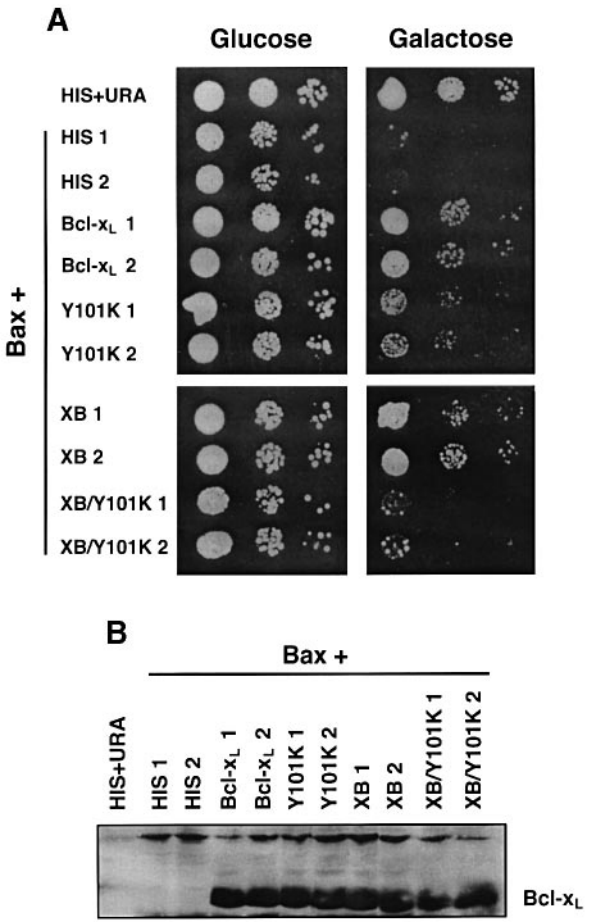

Fig. 5. Bcl-x_L can inhibit Bax-mediated toxicity in yeast by at least two apparently distinct mechanisms. The yeast *S.cerevisiae* was transformed with a multi-copy expression plasmid containing the uracil selection marker and expressing human Bax under the control of a galactose-inducible promoter. Several clones were selected and found to be growth inhibited when Bax expression was induced with galactose. A representative clone was transformed again with a multi-copy expression plasmid containing the histidine selection marker and expressing either human Bcl-x_L or Bcl-x_L mutants, also under the control of a galactose-inducible promoter. Several subclones were selected and characterized, but shown here are representative subclones. (**A**) The ability of Bcl-x_L and Bcl-x_L mutants to suppress Bax-mediated toxicity was determined by growing yeast cells overnight in galactose media and spot-testing on galactose plates starting with an equal number of yeast cells as determined by taking the OD_{600}. Ten-fold serial dilutions were used in the spot-test. Plates were grown at 30°C for 2–3 days. (**B**) An immunoblot to demonstrate Bcl-x_L and Bcl-x_L mutant expression after 18 h of galactose induction.

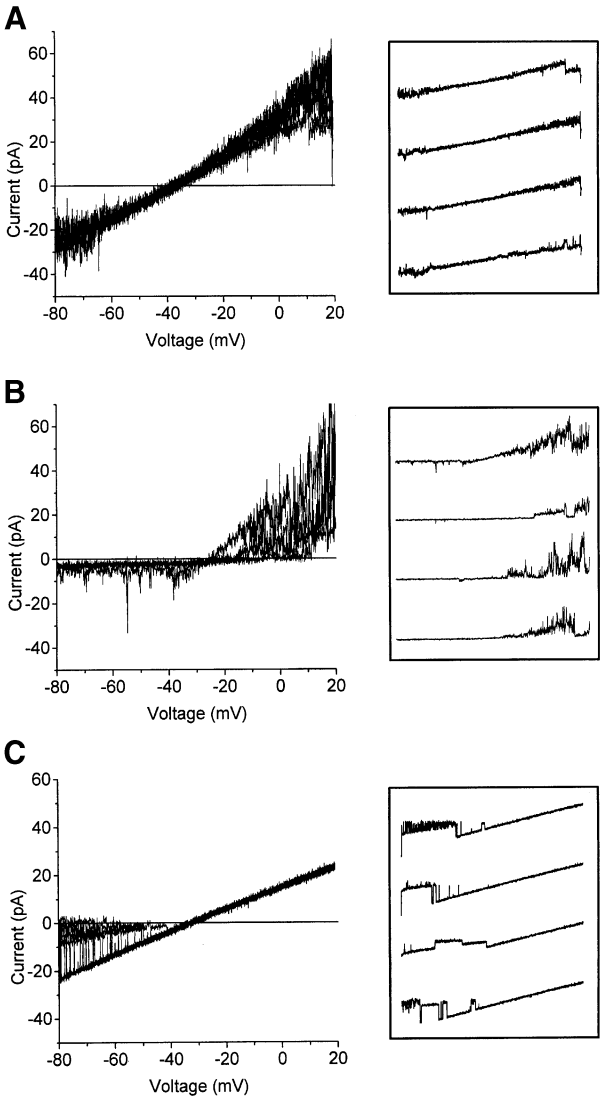

Fig. 6. The XB mutation alters the ion channel properties of Bcl-x_L. (**A**) Recombinant Bcl-x_L or (**B**) and (**C**) XB mutants were added to the *cis* side of a planar lipid bilayer in the presence of a 150:15 mM (*cis*:*trans*) KCl gradient at pH 7.2. After the onset of channel activity, a continuous voltage ramp from –80 to 20 mV or 20 to –80 mV was applied and current fluctuations recorded. Shown on the left is an overlay of several individual current sweeps, and on the right are five representative individual current sweeps displayed separately. The current fluctuations that arise from the XB mutants can display some variation as shown in (B) and (C), with the type of current fluctuations shown in (B) being more common than the type shown in (C). See text for details. The addition of the Y101K mutation to either Bcl-x_L or the XB single mutant did not significantly influence the ion channel properties of the proteins (data not shown).

induced apoptosis in 293 cells, and Bax-induced toxicity in yeast. This implies that all of these pathways share a common feature that can be regulated by ion channel activity. One component of the mammalian apoptotic pathway that Bcl-x_L and Bax may influence and is present in yeast is the mitochondrion. Therefore, changes in mitochondrial properties were compared between mammalian cells undergoing a physiological form of apoptosis and yeast cells expressing Bax.

We have recently shown that in mammalian cells undergoing apoptosis, mitochondria demonstrate increased fluorescence when stained with cationic dyes such as rhodamine 123 (Rh123). This increase in fluorescence is associated with mitochondrial swelling, hyperpolarization, and the cytoplasmic redistribution of mitochondrial pro-apoptotic proteins such as cytochrome *c* (Vander Heiden *et al*., 1997). Figure 8 demonstrates this increase in Rh123 fluorescence in FL5.12 cells induced to undergo apoptosis. In response to growth factor withdrawal, cells transitioned to a population with an increased Rh123 fluorescence prior to cell death. Upon cell death, these cells accumulated in a population with the lowest Rh123 fluorescence (cell viability was measured by propidium iodide uptake; data not shown). As previously reported, the expression of Bcl-x_L prevented the increase in Rh123 fluorescence and enabled growth factor-deprived cells to survive at a decreased mitochondrial membrane potential (Vander

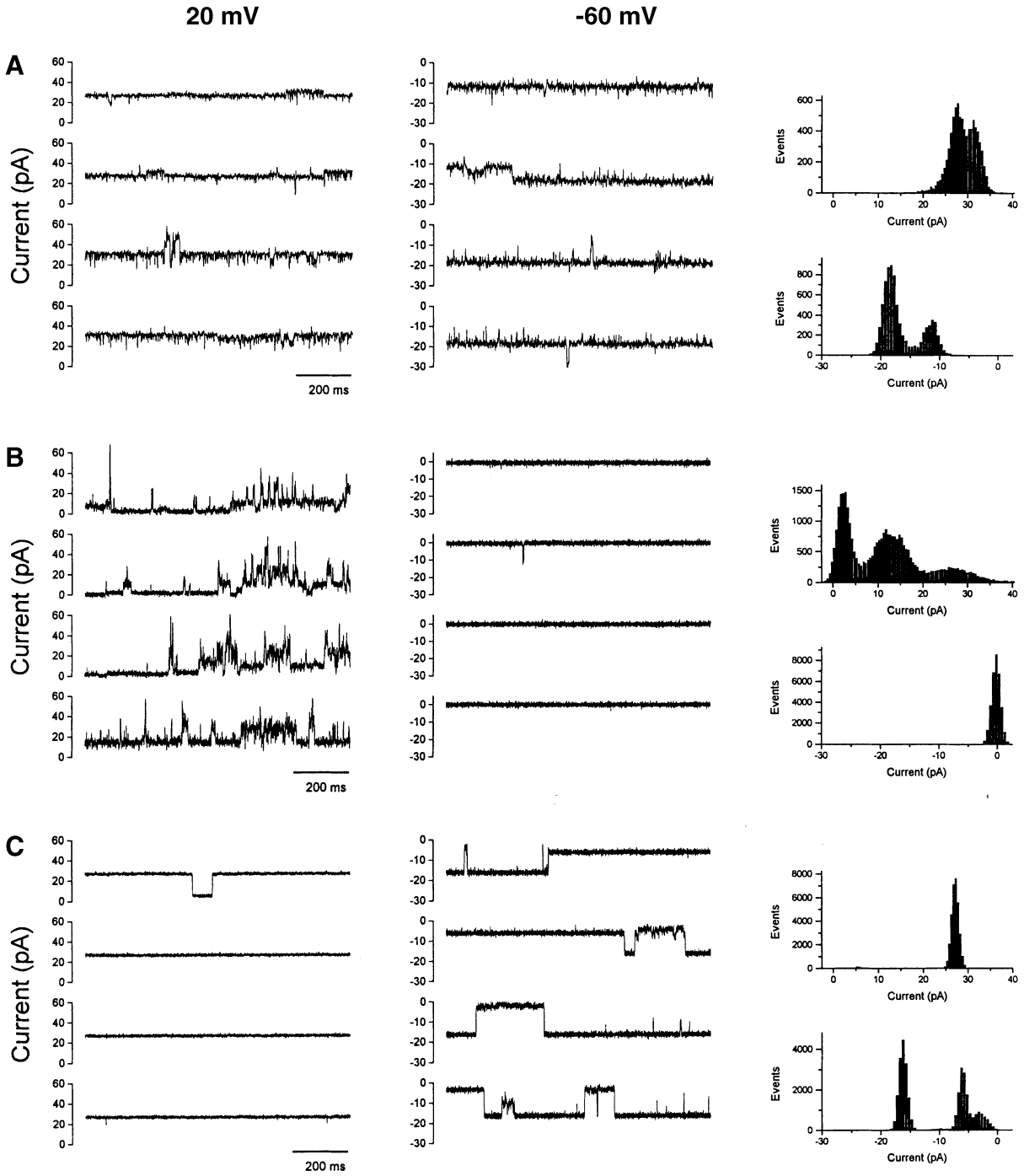

Fig. 7. Sample channel recordings from the XB mutants. (**A**) Recombinant Bcl-x_L or [(**B**) and (**C**)] XB mutants were added to the *cis* side of a planar lipid bilayer in the presence of a 150:15 mM (*cis*:*trans*) KCl gradient at pH 7.2. After the onset of channel activity, a holding potential of either –60 or 20 mV was applied (as indicated) and current fluctuations were recorded. Shown are sample channel recordings at the indicated holding potentials. On the right are current amplitude histograms generated from the channel recordings at each holding potential (20 mV, top; –60 mV, bottom).

Heiden *et al.*, 1997). This decreased potential is reflected in the decreased uptake of Rh123, exhibited by cells cultured in the absence of IL-3. Similar effects were also observed with Bcl-x_L Y101K and XB, suggesting that, like wild-type Bcl-x_L, both mutants can prevent apoptosis by acting at or upstream of events that lead to changes in mitochondrial properties. In contrast, XB/Y101K was significantly impaired in preventing the increase in Rh123 fluorescence. By ~30 h after IL-3 withdrawal, a significant percentage of cells transfected with XB/Y101K had an increase in Rh123 fluorescence, while the Bcl-x_L, Bcl-x_L Y101K and XB mutant cells remained protected from this effect. Even after 72 h of IL-3 withdrawal, no population with a significant increase in Rh123 fluorescence was seen in either the Bcl-x_L, Bcl-x_L Y101K or the XB single mutant transfectants (data not shown).

The increase in Rh123 fluorescence observed in mammalian cells undergoing apoptosis can be recapitulated in

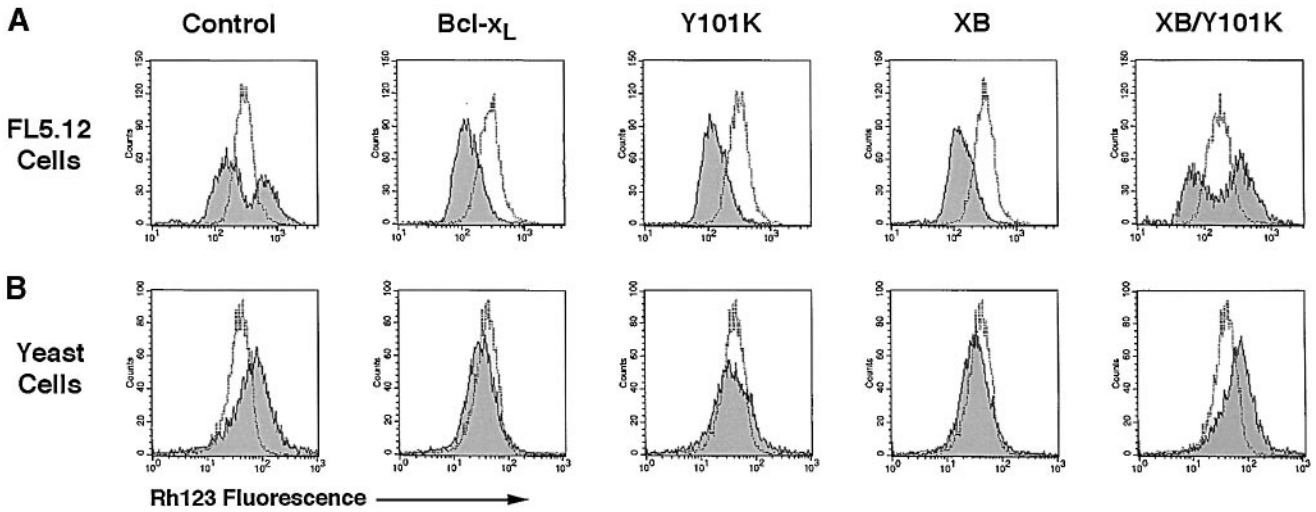

Fig. 8. Apoptosis induction in mammalian cells and Bax expression in yeast both result in an increase in mitochondrial Rh123 fluorescence that is inhibitable by Bcl-x_L but not by XB/Y101K. (**A**) FL5.12 cells were stably transfected with Bcl-x_L, the indicated mutant or an empty expression vector (Control). Cells were grown in the presence (dotted line) or absence (gray fill) of IL-3 and stained with Rh123 at 30 h after growth factor withdrawal. In the case of the control transfectant, Rh123 staining was performed at 18 h. This 12 h delay in the time to increased Rh123 fluorescence is consistent with the cell viability data and may represent a residual capacity of the double mutant to delay apoptosis. (**B**) Yeast cells were transformed with a multi-copy expression plasmid containing the uracil selection marker and expressing Bax under the control of a galactose-inducible promoter. A representative clone was transformed again with a multi-copy expression plasmid containing the histidine selection marker and expressing either Bcl-x_L, Bcl-x_L mutants or an empty vector (Control), also under the control of a galactose-inducible promoter. The double transformants were incubated in minimal media + 3% galactose for 24 h to induce protein expression and stained with Rh123 (gray fill). Yeast cells transformed with the empty expression plasmids were used as a control.

yeast cells expressing Bax (Figure 8). To confirm that increases in mitochondrial dye fluroescence seen in mammalian cells and yeast are not unique to Rh123, other mitochondrial dyes such as JC-1 and TMRE were also tested and found to give similar results (data not shown). These data suggest that Bax can directly influence mitochondrial properties. In contrast to mammalian cells, however, the population of cells with an increased Rh123 fluorescence accumulated and remained stable. One explanation for this may be that yeast cells lack caspases, which in mammalian cells contribute to subsequent mitochondrial depolarization (Susin *et al*., 1997). Regardless of the mechanism, this increase in Rh123 fluorescence was completely inhibited by co-expression of either Bcl-x_L or XB, similar to the results seen with mammalian cells. The non-binding Bcl-x_L Y101K was also inhibitory, albeit to a lesser extent, correlating with its ability to partially reverse Bax toxicity. In contrast, XB/Y101K failed to prevent the Bax-induced increase in Rh123 fluorescence. Together, these data suggest that Bax-induced toxicity in yeast and the apoptotic process in mammalian cells are both associated with changes in mitochondrial properties. The ability of Bcl-x_L to inhibit these common changes can be modulated by mutations that disrupt heterodimerization and by mutations that alter ion channel properties.

Discussion

We have demonstrated that Bcl-x_L can regulate cell survival in the absence of heterodimerization with BH3-containing death agonists such as Bax. Mutants of Bcl-x_L that specifically disrupt critical interactions with BH3-containing proteins retain significant anti-apoptotic function. This heterodimerization-independent mechanism can be disrupted by replacing a 13 amino acid region between helices 5 and 6 with the corresponding region of Bax. Replacement of this region affects the ion channel properties of Bcl-x_L, suggesting that the heterodimerization-independent regulation of cell survival correlates with the ion channel properties of the wild-type protein. Mutation of this region alone does not perturb anti-apoptotic function, suggesting that the disruption of the ion channel properties of Bcl-x_L may not be sufficient to cripple the protective function of the molecule. Together, our data suggests that Bcl-x_L can inhibit apoptosis by at least two mechanisms.

Amino acid substitutions in the XB mutants result in a reversal of the overall charge properties of the substituted region and lead to alterations in Bcl-x_L ion channel properties. Compared with wild-type Bcl-x_L, the ion channels formed by the XB mutants frequently reside at lower conductance states at both positive and at negative potentials. In addition, unlike the wild-type Bcl-x_L channel, the most frequently observed channel formed by the XB mutants is characterized by a voltage-dependent behavior whereby current fluctuations to the open state are favored at positive electrical potentials. Bax has also been reported to form ion channels that display a voltage-dependent behavior, yet the reported properties of Bax-induced membrane permeability appear to be different from that induced by the XB mutants (Antonsson *et al*., 1997; Schlesinger *et al*., 1997). For example, although both the Bax and XB mutant channels exhibit voltage-dependent behavior, conductance by the Bax channel is favored by negative potentials whereas conductance by the XB mutant channel is favored by positive potentials. The XB mutation does not alter the ion selectivity of the Bcl-x_L channel, whereas the Bax channel has been reported to have a selectivity different from that of Bcl-x_L. Thus, the 13 amino acid region of Bax that was utilized to create the XB mutants does not recapitulate many of the ion channel properties

that are associated with Bax channels, nor does it endow Bcl-x_L with Bax function. This region does, however, serve to disrupt the properties of the wild-type Bcl-x_L channel. Attempts to engineer chimeras between Bcl-x_L and Bax that extend beyond the 13 amino acid region resulted in proteins that were poorly expressed and failed to interact with Bax. This was likely due to protein misfolding since amino acids on either side of the 13 amino acid region replaced in creating the XB chimera reside within the interior of the protein (Figure 3A–C).

The XB/Y101K double mutant fails to protect against growth factor deprivation in FL5.12 cells, Bax-induced apoptosis in 293 cells and Bax-induced growth defect in yeast, suggesting that there is a common component to these pathways that may be regulated by the ion channel properties of Bcl-x_L. Furthermore, as in the case of mammalian cells undergoing apoptosis, yeast cells expressing Bax demonstrate changes in mitochondrial function that are inhibitable by Bcl-x_L but not by XB/Y101K. One explanation for these results is that both Bax and Bcl-x_L are able to affect mitochondrial homeostasis through the control of membrane permeability. In mammalian cells, the induction of apoptosis results in mitochondrial membrane hyperpolarization, swelling and the release of cytochrome *c*, the first two of which can be monitored by an increase in Rh123 fluorescence (Vander Heiden *et al*., 1997). Bax expression in yeast also results in an increase in Rh123 fluorescence. Consistent with this, Bax-mediated toxicity in yeast is dependent on mitochondria and leads to the release of mitochondrial cytochrome *c* (Greenhalf *et al*., 1996; Manon *et al*., 1997), an effect that may be directly induced by the protein (Jurgensmeier *et al*., 1998). Thus, Bax is able to disrupt mitochondrial function, leading to apoptosis in mammalian cells and growth inhibition in yeast. Bcl-x_L primarily counters this effect by forming an inactivating heterodimer with Bax in solution. However, when Bcl-x_L is prevented from interacting with Bax, it may form an ion channel that establishes a permeability pathway that counters the effects of the Bax ion channel. The establishment and/or properties of this Bcl-x_L-mediated permeability pathway may be disrupted in the XB mutants. Alternatively, this counter mechanism may involve interactions within the membrane between the central hydrophobic helices of membrane-inserted Bcl-x_L and that of membrane-inserted Bax, resulting in Bax channel inactivation or the formation of a hybrid, non-toxic channel. In this scenario, the XB mutation prevents proper intermembrane helical interactions. Interactions involving membrane-inserted helices are thought to be the mechanism by which immunity proteins prevent the toxicity of bactericidal colicins (Cramer *et al*., 1995; Espesset *et al*., 1996), which contain a pore-forming domain structurally similar to that of Bcl-x_L.

Recently, it has been suggested that Bcl-x_L inhibits apoptosis by binding to CED-4-like molecules such as Apaf-1 to prevent the activation of downstream caspases (Hu *et al*., 1998; Pan *et al*., 1998). Indeed, in mammalian cells, one possible explanation for the heterodimerization-independent effect of Bcl-x_L is that a protective effect of Bcl-x_L is preserved through interactions with other components of the apoptotic machinery that are not disrupted by the mutants used in this study. For example, the mutants of Bcl-x_L used in this study may still dimerize with CED-4-like molecules, downstream caspases or many other proteins that have been identified to interact with Bcl-2 and/or Bcl-x_L. It is difficult to exclude this possibility through the utilization of a mammalian system. However, since homologs to components of the metazoan apoptotic machinery have not been identified in yeast, the effects of Bcl-x_L on Bax-mediated toxicity in yeast presented here and elsewhere (Zha and Reed, 1997) argue that neither interaction with BH3-containing death agonists nor with other components of a metazoan apoptotic machinery are necessary for Bcl-x_L to regulate cell survival. Notably, XB/Y101K still retains some residual protective effect between 24 and 48 h after growth factor withdrawal in FL5.12 cells. In addition, the apoptosis-associated mitochondrial changes in these cells are delayed by ~12 h when compared with control cells. In mammalian cells, this residual protection could be due to the ability of Bcl-x_L to interact with components of the apoptotic machinery. Thus, the regulation of apoptosis by Bcl-2 family members may involve multiple mechanisms. The data presented in this study provide support for this hypothesis and suggest that two of these mechanisms involve heterodimerization with BH3-containing death agonists and the ability to form a sustained ion channel.

Materials and methods

Cell culture, cell transfections and apoptosis assay

The murine prolymphocytic IL-3-dependent cell line, FL5.12, was maintained as described previously (Boise *et al*., 1993). Transfections were performed using 10 μg of plasmid, electroporated into 1×10^7 cells at 960 μF and 250 V. Neomycin-resistant cells were selected in 1 mg/ml of G418. Transfected cells were screened for Bcl-x_L expression by immunoblotting with a rabbit polyclonal antisera to Bcl-x, 13.6.

The human kidney epithelial 293 cell line was maintained in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum, 2 mM penicillin/streptomycin and 10 mM HEPES. For transfections, cells were split to ~20% confluency in a six-well plate. After cells re-adhered, they were transfected by calcium phosphate precipitation, and the medium was changed after 8 h. One microgram of pSFFV-Bax, 4 μg of pSFFV-Bcl-x_L (or Bcl-x_L mutants) and 500 ng of a GFP-expression plasmid (Clontech) were used for each transfection.

Apoptosis was quantitated by propidium iodide exclusion or by measuring the amount of DNA hypodiploidy. For DNA hypodiploidy quantitation, adherent and suspension cells were pooled, fixed with 1% paraformaldehyde for 10 min at room temperature, resuspended in 70% ethanol, and stored at –20°C for at least 1 h. Cells were washed once with phosphate-buffered saline (PBS), resuspended in DNA staining solution (3.8 mM sodium citrate, 0.125 mg/ml RNase A, 0.01 mg/ml propidium iodide), incubated at room temperature for 30 min and analyzed by flow cytometry (Becton Dickinson). The percentage of hypodiploid cells was measured by gating on GFP-expressing cells.

Immunoprecipitations and immunoblotting

For each immunoprecipitation 5×10^6 cells were lysed in 500 μl of 0.2% Nonidet P-40 (NP-40) isotonic lysis buffer (142.5 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM HEPES, 0.2% NP-40, pH 7.4) supplemented with 8 μg/ml aprotinin, 2 μg/ml leupeptin, and 170 μg/ml PMSF. Cellular debris was pelleted by centrifugation at 14 000 *g* for 10 min at 4°C. The supernatant was precleared with 25 μl of protein G–agarose (Gibco-BRL) for 1 h at 4°C on a rocking platform. Two microliters of the anti-Bcl-x mouse monoclonal antibody 7B2 was added to the supernatant and rocked for 6–12 h at 4°C. Twenty-five microliters of protein G–agarose was then added, and the sample was rocked for another hour at 4°C. The agarose beads were spun down and washed three times with 0.2% NP-40 isotonic lysis buffer. The antigens were released and denatured by addition of SDS sample buffer and heating at 95°C for 5 min. *In vitro* translations were done with the TNT Quick system according to the manufacturer's instructions (Promega).

For Bcl-x_L and Bax immunoblotting, samples were separated by 12% SDS–PAGE, transferred to a nylon membrane and blocked with BLOTTO (5% milk + 0.05% Tween 20) for 1 h at room temperature. Membranes were then probed with a mixture of a 1:2500 dilution of 13.6 (anti-Bcl-x rabbit polyclonal antisera) and 0.25 μg/ml of N19 anti-bax rabbit polyclonal antibody (Santa Cruz) in BLOTTO for 1 h at room temperature. The blot was washed 3–4 times for 5 min each in Tris-buffered saline + 0.05% Tween 20 and then probed with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blot was washed as before and developed using enhanced chemiluminescence (ECL; Amersham).

Intracellular staining for flow cytometry

For intracellular staining, approximately 1×10^6 cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. Cells were then washed in wash solution (0.03% saponin in PBS) and resuspended in 100 μl of staining solution (0.3% saponin and 20% goat serum in PBS) along with 1 μl of 7B2, an anti-Bcl-x monoclonal antibody, for 30 min at 4°C. Cells were then washed twice with wash solution and resuspended in 100 μl of staining solution without goat serum along with a 1:100 dilution of a phycoerythrin (PE)-conjugated anti-mouse IgG antibody (Sigma). Staining using the secondary antibody was done for 30 min at 4°C. Cells were then washed twice with wash solution and resuspended in FACS buffer (0.1% sodium azide and 1% bovine serum albumen in PBS). Cells were then analyzed by flow cytometry.

Yeast studies

All studies were done on *S.cerevisiae* strain W303 (ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1). Yeast strains were maintained on synthetic complete media (SC) or minimal media (MM). Human cDNAs for Bax and Bcl-x_L (or Bcl-x_L mutants) were cloned into the multicopy expression plasmid pRS426 GALL (uracil selection) or pRS423 GALL (histidine selection), respectively. These expression plasmids are controlled by the GALL galactose inducible promoter. Yeast were transformed with the Bax expression plasmid by the lithium acetate method and selected on uracil-deficient media. Several clones were characterized, but a single representative clone was then transformed again with Bcl-x_L (or Bcl-x_L mutant) expression plasmids and selected on uracil- and histidine-deficient media. For Bax-toxicity assays, yeast strains containing the Bax plasmid were grown at 30°C overnight in MM + 2% glucose, washed three times in MM + 3% galactose, and diluted 1:20 in MM + 3% galactose. After incubating for an additional 16–18 h, 5 μl of cells at a concentration equal to an OD_{600} of 0.2 was spotted onto either MM + 2% glucose plates or MM + 3% galactose plates. Plates were incubated at 30°C for 2–3 days.

To prepare whole-cell lysates for immunoblotting, yeast strains were grown for 18 h in MM + 3% galactose. Cells were harvested, washed three times in 50 mM Tris–HCl + 2 mM EDTA pH 7.5, and resuspended in three cell volumes of RIPA (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5) supplemented with 170 μg/ml PMSF, 0.5 mM TPCK, 0.025 mM TLCK and 1 μM pepstatin. An equal volume of acid-washed glass beads was added and cells were vortexed for 10 min at 4°C. The lysates were then cleared by centrifugation at 14 000 *g* for 5 min at 4°C.

Protein preparation and NMR structure determination

Recombinant human Bcl-x_L proteins containing a six amino acid C-terminal histidine tag was expressed in *Escherichia coli* strain HMS174(DE3) or BL21(DE3) and purified by affinity chromatography on a nickel-IDA column (Invitrogen) as described previously (Muchmore *et al*., 1996). NMR spectra were acquired as described previously (Sattler *et al*., 1997).

Planar lipid bilayer studies

Planar lipid bilayers were formed across a 200 μm diameter aperture in the wall of a Delrin cup. Lipid bilayer-forming solution contained 30% phosphatidylserine and 70% phosphatidylcholine (Avanti Polar Lipids) at a concentration of 50 mg/ml in *n*-decane. Protein was added to one side of the bilayer, defined as *cis*. The concentration of protein added was in the range of 0.3–3 μg/ml. The other side of the bilayer was defined as *trans* and was the virtual ground. Solutions contained 150:15 mM KCl (*cis*:*trans*) and were buffered at pH 7.2 with 10 mM HEPES. Both the *cis* and the *trans* compartments were connected to separate chambers containing a $AgCl_2$ electrode by a KCl bridge. Current fluctuations were measured using an Axopatch 200B current/voltage conversion amplifier (Axon Instruments). The data were digitized at 2–4 kHz and filtered at 0.75–1.0 kHz using a 12 bit A/D-D/A converter (Axon Instruments) and a 8-pole Bessel filter. Data were acquired and analyzed using pClamp software (Axon Instruments).

Measurement of Rh123 uptake

FL5.12 cells were incubated for 30 min with 2 μM Rh123 (Molecular Probes) in culture medium at 37°C. Cells were then washed in PBS and resuspended in PI solution (PBS + 2 μg/ml propidium iodide + 1% BSA + 0.01% sodium azide, pH 7.4). Rh123 fluorescence and propidium iodide fluorescence was measured by flow cytometry (Becton Dickinson). For measurement of Rh123 fluorescence in yeast, log-phase cells growing in MM + 2% glucose were washed three times and diluted 1:20 or 1:50 in MM + 3% galactose. After a 24 h incubation at 30°C, cells were resuspended in 10 μg/ml of Rh123 in PBS and stained for 15 min at room temperature. Cells were then sonicated for 5 s to yield a single-cell suspension. Rh123 fluorescence was measured by flow cytometry.

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