The appearance of antigen in animals makes antigen-specific T cells divide and then die (1, 2). It is now well known that the death of these cells is to a large extent governed by members of the Bcl-2 family of proteins (3–6). Overexpression of antiapoptotic members, such as Bcl-2 and Bcl-xl (the Protectors), inhibits activated T cell death (7). Activated T cell death is likewise inhibited by double deficiencies in the Executioner members, Bak and Bax, which are supposed to actually kill the T cells (8, 9).

A third group of proteins in this family, the so-called Messengers, also affect T cell death, and deficiencies in one of these proteins (Bim) also protect T cells against death (2, 10).

The fact that members of three different groups of Bcl-2-related proteins affect activated T cell death leads to difficulties in predicting the exact course of events that kills or protects the cells. It is agreed that the Protectors somehow prevent death and the Messengers and Executioners drive death. What is not clear, however, is which proteins must interact with which to control these events. Three models have been suggested. In the first model, the Protectors are supposed to be bound to the Messengers in healthy T cells and the Executioners are in some innocuous form. After activation, as the T cells approach death, it is suggested that some of the Messengers are released from the Protectors and that the freed proteins directly or indirectly activate the killing properties of the Executioners (11–15). In a second model, the Protectors are bound to the Executioners in healthy cells. When the activated T cells approach death, the ratio of these proteins changes such that the Executioners are released from the protective custody of the Protectors and, thus freed, are able to kill the cell (12, 16). Finally it is possible that the Executioners are released to kill the cell via some event that does not directly involve binding to either a Protector or Messenger (17).

The experiments described here test the second of these possibilities by examining whether the Protectors bind the Executioners in healthy cells. We show that the Protector Bcl-2 does not coprecipitate with Bak or Bax...
even under conditions in which the Executioners are activated by incubation in NP-40. Such coprecipitation experiments could not be interpreted with Bcl-xl because we could not efficiently immunoprecipitate all the protein from normal cells. Thus, in a different approach to the problem, we tested the ability of a tagged variant of Bcl-xl lacking its flexible loop (Bcl-xlΔLoop; references 18–20) to coisolate with Bax or Bak after cosynthesis in insect cells. Normal Bcl-xl coprecipitated with both of these proteins; however, Bcl-xlΔLoop did not. However, like normal Bcl-xl, Bcl-xlΔLoop bound the Messenger protein Bim. In spite of its inability to bind Bak or Bax, Bcl-xlΔLoop protected T cells from death as efficiently as the wild-type protein. Thus, these experiments suggest that, in activated T cells, the Protectors act by binding to Messengers and not by engaging the Executioners directly.

A byproduct of these experiments is a higher resolution structure, by X-ray crystallography, of the structure of Bcl-xl bound to a peptide from Bim. This structure differs from those that have been previously reported (20–22) in that it reveals the configuration of a few more of the residues of the flexible loop (amino acids 22–82) of Bcl-xl. Engagement of Bcl-xl by Bim renders a site in the loop sensitive to proteolytic cleavage and changes the conformation of the loop amino acids that can be resolved. Moreover, Bcl-xlΔLoop binds Bim less well than wild-type Bcl-xl does. Thus, the loop domain of Bcl-xl may play a role in some currently unknown function of Bcl-xl.

**RESULTS**

**Bcl-xl lacking its loop domain protects activated T cells from death**

The structure of Bcl-xl has been solved by several groups (20–22). However, the complete structure of the protein is not known. In all reports about Bcl-xl, a portion of the protein stretching from about amino acids 28–82 could not be resolved. To study the contribution of this part of Bcl-xl to the protective activity of the protein, we activated T cells in vivo with the Vβ8-engaging superantigen, staphylococcal enterotoxin B (SEB), isolated the cells 2 d later, and studied the ability of transduced wild-type Bcl-xl or Bcl-xlΔLoop to protect the activated T cells against death. As shown in Fig. 1 A, both constructs of Bcl-xl protected the cells very well against death. Unfortunately, because only ~5% of the T cells were transduced in this and similar experiments, we could not evaluate directly how much the Bcl-xl transduction had increased levels of Bcl-xl in the T cells. Therefore, to get some estimate of these values, we compared the levels of Bcl-xl in the Phoenix cells that had been transduced either with the control plasmid, expressing plasmids coding for intact Bcl-xl or Bcl-xlΔLoop. Staining showed that between 21 and 25% of the cells were transduced with each plasmid. The cells were lysed and Bcl-xl was immunoprecipitated with an antibody that reacts with both human and mouse Bcl-xl. The immunoprecipitates and supernatants were run on SDS-PAGE and Western blotted for Bcl-xl. Anti–Bcl-xl precipitated Bcl-xl very inefficiently, a consistent finding in our experiments (Fig. 1 B) and one that is common to all anti–Bcl-xl antibodies we have used. Nevertheless, by combining the data from the immunoprecipitates and supernatants we could get an estimate of the increase in Bcl-xl afforded by the Bcl-xlΔLoop expressing plasmids.

The estimates of Bcl-xl expressed by the transfected genes varied somewhat depending on the detergent used, and was between 40–100% of endogenous levels in Phoenix. Bcl-xl and Bcl-xlΔLoop were expressed equally well (Fig. 1 B).

**Bcl-2 does not coprecipitate with Bak or Bax from activated T cells**

Activated T cells transduced with retroviruses expressing Bcl-2 are protected from death, like the cells transduced with retroviruses expressing Bcl-xl or Bcl-xlΔLoop. To test the idea that Bcl-2 protects activated T cells from death by binding to the Executioners Bak and Bax, Bcl-2 was precipitated from CHAPS or NP-40 lysates of resting and activated T cells, and the precipitates were analyzed by Western blot for coprecipitation of...
Bak or Bax. Immunoisolates of Bcl-2 contained neither Bak nor Bax (Fig. 2 A). A repeat of this experiment showed that the activated T cells were indeed destined to die more rapidly than the naive cells (Fig. 2 B) and confirmed that Bcl-2 was not bound to Bax in either resting or activated T cells but was bound, as we have reported before, to Bim in both types of cells (reference 23; Fig. 2 C). Similar experiments with Bak or Bax. Immunoisolates of Bcl-2 contained neither Bak nor Bax (Fig. 2 A). A repeat of this experiment showed that the activated T cells were indeed destined to die more rapidly than the naive cells (Fig. 2 B) and confirmed that Bcl-2 was not bound to Bax in either resting or activated T cells but was bound, as we have reported before, to Bim in both types of cells (reference 23; Fig. 2 C). Similar experiments with immunoprecipitates of Bcl-xl were tried with no consistent sign that Bcl-xl was bound to either of the Executioners in healthy cells (unpublished data). However, under no circumstances could we precipitate all the Bcl-xl from the cells. As illustrated in Fig. 1 B, only a maximum of 30% of the Bcl-xl could be precipitated, even with use of polyclonal antibodies under the best of circumstances. Therefore, we could never be sure that we had isolated the fraction of Bcl-xl that might be associated with Bak or Bax, should such complexes exist. To find out whether or not Bcl-xl bound Bak or Bax in healthy T cells, we therefore turned to a different type of experiment.

Unlike wild-type Bcl-xl, Bcl-xlΔloop cannot bind a truncated, conformationally altered form of Bax

Given our failure to immunoprecipitate Bcl-xl completely from T cells and thus examine its intracellular association with the Executioners, we decided to test the idea that Bak and Bax bind Bcl-xl using insect cells. To perform the test, Bcl-xl or Bcl-xlΔloop were expressed in insect cells, together with Bak or 6-His−tagged Bak. The insect cells were lysed with NP-40, a detergent, which, like other powerful nonionic detergents, converts innocuous Bak or Bax into their N-terminal−exposing, death dealing forms that have previously been shown to bind Bcl-xl or Bcl-2 (24, 25). Bak was precipitated with the anti−BaxNter monoclonal antibody, 6A7, that reacts with the supposed death−dealing form of Bak (24).
are the results of immunoprecipitates of $12.5 \times 10^6$ Phoenix cells or $10^7$ precipitates and their supernatants were Western blotted for Bax. Shown body 6A7. Bax immunoprecipitated by 6A7 is here termed DeN. Immunoisolation of Bax from the NP-40 lysates readily cocoprecipitated Bcl-xl. Bcl-x$\Delta$Loop, however, did not coisolate with Bax. Likewise, Bak coprecipitated with wild-type Bcl-xl but not with Bcl-x$\Delta$Loop (Fig. 3). To check that this was not some artifact caused by the environment inside insect cells, we repeated the experiment with transfections in Phoenix cells (Fig. 3 C). Again, Bax coisolated with Bcl-xl but not with Bcl-x$\Delta$Loop. As expected, Bax did not cocoprecipitate with Bcl-xl if the Phoenix cells were lysed in CHAPS rather than NP-40 (unpublished data). This result was reflected in the degree of conformational change of Bax by NP-40, with virtually none of the cellular Bax reacting with the anti-conformationally changed Bax antibody 6A7 after Phoenix of T cell lysis in CHAPS and up to 70% of the Bax reacting with 6A7 after lysis in NP-40 (Fig. 4).

To demonstrate that the Bcl-x$\Delta$Loop was functional in insect cells, we coexpressed, in the cells, Bcl-xl or Bcl-x$\Delta$Loop together with Bim, a protein to which Bcl-xl binds well. Both forms of Bcl-xl were associated with Bim, although less Bcl-x$\Delta$Loop bound to Bim than Bcl-xl did, especially in the case of BimEL (Fig. 5).

Thus, Bcl-x$\Delta$Loop does not bind Bak or Bax, even when the latter proteins are conformationally changed to their supposed death-dealing form. Nevertheless, Bcl-x$\Delta$Loop protects activated T cells from death, suggesting that interaction of Bcl-xl with conformationally altered Executioners is not important to the protective effects of Bcl-xl.

The flexible loop of Bcl-xl is affected by binding of Bcl-xl to Bim

These experiments led us to be curious about the functions of Bcl-xl’s flexible loop. Somehow this loop affects the ability of Bcl-xl to bind the conformationally changed forms of the Executioners, even though this interaction is not important in the ability of Bcl-xl to protect T cells from death. Also, the loop modestly affects the ability of Bcl-xl to bind intact Bim, even though the major site of this interaction is thought to be via the engagement of Bim’s Bcl-2 homology 3 (BH3) region by the BH3 binding groove of Bcl-xl, a site on Bcl-xl that does not include Bcl-xl’s flexible loop.

To address the function of Bcl-xl’s loop, we made another attempt to resolve its structure. To this end, hanging drops containing Bcl-xl and Bim were set up, with each protein lacking only its transmembrane region. Crystals appeared. Their characteristics, shown in Table I, revealed some differences between these crystals and those we have previously reported between Bcl-xl and a fragment of Bim (22). Most notably, the new crystals diffracted to a 1.2-Å resolution, allowing a much higher quality electron density map and analysis of the complex (Fig. 6 A).

As before, the crystals contained Bcl-xl bound to a peptide of Bim. The Bim protein had, once again, been degraded by proteolysis during the time the crystals took to form (Fig. 6 B). In general, the combination of Bcl-xl in this new complex was very similar to that previously reported (20-22) with the exception that the Bim peptide was somewhat shorter at both ends than that which we observed in our previous structure (Fig. 6 C). This allowed for a change in the crystal packing, accounting for the changes in crystal parameters between this and our previous paper (22). Nevertheless, the Bim peptide was bound to Bcl-xl very much as before with hydrophobic and hydrophilic interactions allowing a very stable complex and similar changes in the structure of Bcl-xl by comparison with its unengaged fold.
Most of the loop region between Bcl-xl’s α1 and α2 helices remained unresolved in this new structure. The higher resolution of these crystals allowed us to see some of the amino acids at the beginning and end of the loop that had not previously been visible in complexes between Bcl-xl and BH3 peptides, but that were, to some extent, seen in the structure of Bcl-xl alone (20–22). Thus our new structure showed that the conformation of the N-terminal end of the Bcl-xl loop domain is changed by binding to Bim (Fig. 7, A and B). In free Bcl-xl, a hydrophobic pocket formed between Bcl-xl’s α1 and α5 helices is occupied by Phe27, whereas in the Bcl-xl–Bim BH3 complex, the pocket is occupied by Trp24. Likewise, in free Bcl-xl the stretch of amino acids 22–28 has an extended helix-like conformation, whereas this region is completely linearized in the Bcl-xl–Bim BH3 complex. At the other end of the loop, clear density and low temperature factors can be seen for amino acids 79–82 and, in the complex, these amino acids are interacting stably with the body of Bcl-xl (Fig. 7 C). The easy identification of these amino acids in the complex, but not in free Bcl-xl, suggests that they have changed position in the complexed versus free Bcl-xl and are now attached more intimately to the rest of the protein.

**Engagement of Bim makes Bcl-xl’s loop susceptible to proteolysis**

Even at the high resolution of our new crystal, electron density for the loop faded gradually after Phe27, indicating that the rest of the loop is relatively unstructured. At the C-terminal end of the loop, however, electron density reappeared abruptly at Glu79, suggesting that in the crystal Bcl-xl may have been cleaved at this point (Fig. 7). To test this, we ran the protein in the crystal on SDS-PAGE and found fragments that were consistent with proteolysis of Bcl-xl (Fig. 8). Such fragments were not seen in the preparation of Bcl-xl before it was set up to crystallize, nor in crystallization drops set up at the same time as those analyzed in Fig. 8, but containing Bcl-xl without Bim. Because all the proteins were purified similarly.

### Table I. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
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<tr>
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<tr>
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<tr>
<td>Average I/σ</td>
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<td>Rmerge(%)³</td>
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**Refinement**

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<td>Rworking (%)²</td>
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<td>Rfree (%)</td>
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**Average B factors (Å²)**

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<td>Allowed regions</td>
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<tr>
<td>Generously allowed regions</td>
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<tr>
<td>Disallowed regions</td>
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<tr>
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<td>B factor side chain (Å²)</td>
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<tr>
<td>Cross-validated coordinate error (Å)</td>
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</tbody>
</table>

³Rmerge = Σ(|I – <I>|IΣ I|I)
⁴Excluding glycine and proline.

---

**Figure 6. The overall structure of Bcl-xl–Bim.** (A) The 2Fo-Fc electronic density (1.4σ cutoff) of the BH3 region of the Bim peptide. Three conserved BH3 hydrophobic amino acids, L94, I97, and F101, are labeled. (B) A ribbon representation of the complex of Bcl-xl and the Bim fragment. The eight α helices of Bcl-xl are labeled and colored sequentially: yellow, orange, red, blue, green, cyan, magenta, and purple. (C) A superposition (Swiss PDB Viewer [reference 47]) of the current and previous (available from GenBank/EMBL/DDBJ under accession no. 1PQ1) Bcl-xl–Bim complexes.
from insect cell supernatants, and thus had the same chance of contamination with insect cell proteases, we concluded that engagement of Bcl-xl by Bim may have rendered the flexible loop of Bcl-xl susceptible to proteolysis.

**DISCUSSION**

In spite of many years’ work, we still don’t know exactly how the Executioners Bak and Bax are triggered to kill activated cells. The work described here suggests that in T cells triggering does not occur because of release of Bak and Bax from the embrace of Protectors such as Bcl-2 and Bcl-xl. We can see no evidence that Bcl-2 binds the innocuous forms of Bak or Bax in T cells. Bcl-xl in both its intact and truncated (Bcl-xlΔLoop) form protects T cells from death. Nevertheless, Bcl-xlΔLoop does not bind the Executioners, even after they have been converted by incubation in NP-40 to their cell-killing forms (24). One could argue that the latter experiment was done only in insect or Phoenix cells and not in the environment of interest, i.e., in activated T cells, where conditions may be different. Although this is possible, we think it unlikely, especially because in the insect cells, for example, both Bcl-xl and Bcl-xlΔLoop are well able to bind a verified partner (Bim) for these proteins.

It could be that Bcl-xlΔLoop does not function in the same way as normal Bcl-xl. For example, both proteins may bind Bim, as shown here, but denatured Bax and Bak may be able to displace Bim from Bcl-xl but not from Bcl-xlΔLoop. If this were correct, one might expect Bcl-xlΔLoop to be a better Protector than Bcl-xl. However, that doesn’t seem to be the case. Activated T cells appear to be equally well protected by the two proteins (Fig. 1), suggesting that the two proteins act similarly.

The involvement of Bcl-xl’s flexible loop in the binding of the protein to Bax after cell solubilization in NP-40 has been studied previously (19) in the paper that first investigated the functions of the loop. Chang et al. (19) reported that Bcl-xl lacking its loop continued to bind Bax, a result that is exactly the opposite to that reported here. There are several differences in methodology and cell type that could account for the discrepancy. One explanation stems from the fact that in the previous paper, the band that coprecipitated with Bcl-xlΔLoop was identified as Bax on the basis of its molecular weight, not by Western blot with a specific antibody. Bax and Bim have approximately the same molecular weight, and the band thought to have been Bax could have been some other protein of similar molecular weight, e.g., Bim, a protein which is known to bind Bcl-xl (15, 23).

The function of Bcl-xl’s flexible loop, and the comparable structure on Bcl-2, has long been a mystery. Several papers have shown that the ability of these proteins to protect cells from death is more or less unaffected by absence of their loops (18, 26, 27), which is a result we confirm here. Indeed, sometimes the absence of the loop improves the antiapoptotic activities of the proteins (19, 28). This may be because serines on the loops can be phosphorylated, and such...
phosphorylation inhibits the protective and increases the death-dealing properties of the proteins (28–30).

It has also been reported that asparagines in the flexible loop of Bcl-xl can be deamidated in tumor cells in response to DNA damage (31, 32) and that deamidation renders Bcl-xl less able to block the proapoptotic activity of BH3-only messenger proteins. This result suggests a relationship between Bcl-xl’s engagement of a Messenger protein such as Bim and the structure of Bcl-xl’s loop. As such, the result is reminiscent of our finding that although Bim engages Bcl-xl very well via binding of its BH3 region to the BH3 binding groove of Bcl-xl, somehow engagement of Bim’s BH3 affects the structure of Bcl-xl’s flexible loop.

Finally, caspase-dependent cleavage of the flexible loop of Bcl-xl, a phenomenon that converts Bcl-xl into a proapoptotic protein (33, 34), has been described. Interestingly, one of the cleavage sites reported is only two amino acids away from Glu79, the amino acid at the N terminus of one of the Bcl-xl fragments we found in our crystal structures. Perhaps the protein is particularly sensitive to proteolysis in this region, particularly after engagement of Bim.

In summary, the data presented here suggest that the Executioners are not kept in check by binding to antiapoptotic proteins. How then are the Executioners triggered? Most likely, in activated T cells, this process begins when, as antigen disappears from animal, levels of Bcl-2 in the activated T cells fall without a concomitant fall in levels of Bim (35). In healthy T cells, most of the Bim in the cell is bound to Bcl-2 or Bcl-xl (23). We predict that when Bcl-2 levels fall in T cells, some Bim is released. The free Bim then directly or indirectly causes Executioner activation.

MATERIALS AND METHODS
Measurement of the antiapoptotic activity of Bcl-xl and Bcl-xlΔLoop. Constructs of Bcl-xl and Bcl-xlΔLoop (Bcl-xl lacking amino acids 45–84; reference 21) were cloned into a plasmid version of the Thy-1.1 expressing retroviral construct MIT (38) and cotransfected by standard methods with pCL-Eco (a plasmid vector expressing the structural proteins of Moloney murine leukemia virus; a gift of I. Verna [Salk Institute, La Jolla, CA [reference 39]]) into the packaging cell line Phoenix-Eco. Activated T cells were purified from the lymph nodes of mice expressing a Vß8+ transgene, VßD0 (23), which had been treated 2–3 d before with 100 μg of mouse SEB, which is a Vß8-specific superantigen that activates all the T cells in VßD0 mice. The activated T cells were transduced with retrovirus by centrifugation at 1000 g for 2 h, followed by washing and incubation at 37°C in Dulbecco’s enriched culture medium. In some experiments, death of the cells was slowed by maintaining them in the cold overnight after the spinning and before culture at 37°C. At various times thereafter, the cells were stained with antibodies against Vß8, Thy-1.1, and either CD4 or CD8 and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). The viability of Thy-1.1+/Vß8+ cells bearing either CD4 or CD8 was determined by FSC/SSC gating (38, 40).

Use of mice for these experiments has been approved by the National Jewish Medical and Research Center Animal Care and Use Committee.

Analysis, by immunoprecipitation, of association between Bcl-2 and Bak or Bax in T cells. T cells were purified from VßD0 mice, which were untreated or had been treated 2 d before with 100 μg SEB to yield resting and activated cells, respectively. The cells were lysed with solutions containing protease inhibitors and 2% CHAPS or 0.5% NP-40 and spun at 14,000 rpm in a microfuge for 15 min to remove nuclei and unsheared cells. The lysates were incubated overnight with beads coupled to hamster anti–Bcl-2, 3F11 (41). The beads were then extensively washed. Bound proteins were released by boiling in SDS loading buffer, run on 10–20% gradient gels (Bio-Rad Laboratories), and Western blotted with mouse anti–human and mouse Bcl-2 (Zymed Corp.), rabbit anti-Bak NT (Upstate Biotechnology), or rabbit anti-Bax N-20 (Santa Cruz Biotechnology, Inc.). Secondary antibodies were horseradish peroxidase–coupled donkey anti–mouse IgG or donkey anti–rabbit IgG (The Jackson Laboratory). In experiments in which we failed to satisfactorily immunoprecipitate, Bcl-xl lysates were tumbled overnight with excess beads bearing two different hamster anti–Bcl-xl antibodies: 2H12 (24) or Ham151–297.5 (unpublished data) or rabbit polyclonal anti–Bcl-xl (Transduction Laboratories). Bcl-xl was Western blotted with rabbit anti–Bcl-xl (Transduction Laboratories) followed by donkey anti–rabbit IgG (The Jackson Laboratory).

Analysis by immunoprecipitation of the association of Bcl-xl or Bcl-xlΔLoop with Bak or Bax. Baculoviruses encoding Bak, Bax, 6-His–tagged Bcl-xl lacking its transmembrane region (Bcl-xlΔMem), or lacking both its transmembrane region and its flexible loop (amino acids 45–84 [Bcl-xlΔLoopΔMem]) were constructed as previously described (22, 42). High Five insect cells (Invitrogen) were infected with viruses expressing either Bak or Bax and with viruses expressing one of the forms of Bcl-xl. 4 d later the cells were lysed in Ni-NTA binding buffer by sonication. 0.5% NP-40 was added to the lysis buffer to increase the likelihood that Bcl-xl would bind Bak or Bax (24). After centrifugation (13,000 g for 60 min), supernatants were incubated with beads bearing an antibody to activated Bak (6A7 [reference 23]) or Ni-NTA beads for 1 h in 4°C. After thorough washing using the binding buffer, complexes were isolated by boiling in SDS loading buffer, run on SDS-PAGE, and Western blotted as described in the previous paragraph.

To study the interaction of Bcl-xl and Bax in Phoenix cells, Bax was cloned into the MIT plasmid (38). Phoenix cells were cotransfected with the Bax plasmid and the MIT plasmid encoding either Bcl-xl or Bcl-xlΔLoop as described in Measurement of the antiapoptotic activity..., except that the pCL-Eco helper plasmid was omitted. Transfected cells were incubated at 37°C. After 24 h, their supernatants were replaced with new Dulbecco’s enriched culture medium. After another 24 h, cells were harvested and the cell pellets were lysed with 0.5% NP-40 in PBS buffer (50 mM NaH2PO4, Na2HPO4, and 150 mM NaCl, pH 7.5). The lysates were cleared by
centrifugation at 10,000 g for 30 min. Bax was immunoprecipitated from the lysate with the 6A7 monoclonal antibody (24) and subjected to SDS-PAGE. Any Bcl-xL associated with the immunoprecipitated Bax was detected by blotting the gel with anti-Bcl-xL antibody.

Coprecipitation of Bim with Bcl-xL or Bcl-xLΔloop. Baculoviruses encoding BimEL−6-His, BimL−6-His, BimELΔMem−6-His, and BimLΔMem−6-His were constructed and used to coinf ect insect cells with viruses expressing Bcl-xLΔLoop or Bcl-xLΔLoopΔMem. 4 d later, complexes were isolated from the insect cells. Equal cell equivalents of every sample were loaded, separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with an anti–Bcl-xL antibody (Santa Cruz Biotechnology, Inc.).

Preparation of the complex of Bcl-xL and Bim. The CDNA fragment encoding amino acids 1−196 of mouse Bcl-xL was cloned under the control of the polyhedron promoter into a previously described baculovirus transfer plasmid (22, 42). Active virus was made by cotransfection into SF9 insect cells (Invitrogen) with the plasmid and BacVector 3000 baculovirus DNA (Novagen) by the calcium phosphate coprecipitation method. A high titered virus stock was made by further propagation in SF9 cells. Virus producing BimL with an additional C-terminal 6-His tag was made by the same method (22). The C-terminal membrane interaction domain of BimL was truncated to increase the solubility of the protein (BimLΔMem).

To prepare the complex of Bcl-xL and Bim, High Five insect cells were coinfected with the two viruses and cultured for 24 h at 27°C. The infected cells were then transferred to 19°C and harvested by centrifugation (1,500 g for 10 min) 4 d later. The cells were lysed by sonication in Ni-NTA binding buffer (50 mM NaH2PO4, 300 mM NaCl, and 5 mM imidazole, pH 8.0). Lysates were cleared by high-speed centrifugation (100,000 g for 60 min), and proteins were purified from the supernatant using Ni-NTA columns (QiAGEN). The Bcl-xL−Bim complex was eluted from the column with 500 mM imidazole and purified to Superdex 200 size exclusion column (GE Healthcare) in PBSA buffer (50 mM NaH2PO4, Na2HPO4, 150 mM NaCl, and 5 mM Na2EDTA, pH 7.5). The complex was further purified using ion exchange chromatography on a Mono-Q column (GE Healthcare) and a Tris-NaCl gradient (25 mM Tris, 100−1,000 mM NaCl, pH 8.2). The complex was concentrated to 15 mg/ml in Tris-NaCl buffer (10 mM Tris and 100 mM NaCl, pH 8.2) for crystallization.

Crystallization and data collection. Crystallization trays were set up using the hanging drop vapor diffusion method. Equal amounts of protein solution and mother liquor (25% PEG4000, 0.1 M NaAc, pH 4.6, and 0.2 M (NH4)2SO4) were mixed in the hanging drops. After ~11 mo at 4°C, crystals appeared. The crystals were flash-frozen in liquid nitrogen after brief transfer to a solution containing the mother liquor supplemented with 20% glycerol. A 1.2-Å high-resolution diffraction dataset was collected under cryogenic conditions at beam line SBC 19BM at Advanced Photon Source at Argonne National Laboratory. The data were processed with HKL2000 package (43).

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