The structure of human BCL-w, an anti-apoptotic member of the BCL-2 family, was determined by triple-resonance NMR spectroscopy and molecular modeling. Introduction of a single amino acid substitution (P117V) significantly improved the quality of the NMR spectra obtained. The cytosolic domain of BCL-w consists of 8 α-helices, which adopt a fold similar to that of BCL-xL, BCL-2, and BAX proteins. Pairwise root mean square deviation values were less than 3 Å for backbone atoms of structurally equivalent regions. Interestingly, the C-terminal helix α8 of BCL-w folds into the BH3-binding hydrophobic cleft of the protein, in a fashion similar to the C-terminal transmembrane helix of BAX. A peptide corresponding to the BH3 region of the pro-apoptotic protein, BID, could displace helix α8 from the BCL-w cleft, resulting in helix unfolding. Deletion of helix α8 increased binding affinities of BCL-w for BAK and BID BH3-peptides, indicating that this helix competes for peptide binding to the hydrophobic cleft. These results suggest that although the cytosolic domain of BCL-w exhibits an overall structure similar to that of BCL-xL and BCL-2, the unique organization of its C-terminal helix may modulate BCL-w interactions with pro-apoptotic binding partners.

Programmed cell death or apoptosis is an essential and integral part of development and tissue turnover under normal physiological conditions and perturbations to this process result in various diseases ranging from autoimmune disorders to cancer (1–4). Apoptosis is generally regulated by the fine balance and interplay among members of the BCL-2 family of proteins in a cell and tissue specific manner (5, 6). This family comprises nearly 20 proteins, which share one or more short regions of sequence homology, the BCL-2 homology regions (BH1, BH2, BH3 and BH4) that are essential for their function (5). The family is further divided into three subfamilies: anti-apoptotic members including BCL-2, BCL-xL, BCL-w, MCL-1, and A1, which contain all four BH regions; pro-apoptotic members that contain BH1, BH2 and BH3 regions and function as death effectors (BAX and BAk); and pro-apoptotic BH3-only members (e.g. BAD, BID, BIM, BIK), which couple upstream death stimuli, such as tumor necrosis factor receptor stimulation or p53-mediated stress signals, to regulation of the multi-BH region members (7). The ratio of anti-apoptotic versus pro-apoptotic members often determines cell death or survival in response to these signals, and alterations in these ratios can result in disease susceptibility. Enhanced expression of anti-apoptotic members occurs in many cancers. For example, up-regulation of BCL-2 in follicular lymphoma, leukemia, breast, and lung cancers (8–11), BCL-xL in liver cancer, myeloid and T-cell leukemias (9), MCL-1 in multiple myeloma (12) and ovarian carcinoma (13), and BCL-w in colorectal carcinomas (14) has been observed and likely contributes to both the etiology of these cancers and the development of chemoresistance, which is frequently encountered. To date, the solution structures of BCL-xL (15, 16), BCL-2 (17, 18), BID (19, 20), and BAX (21) have been determined and surprisingly they all possess very similar three-dimensional structures despite of their opposing roles in the regulation of apoptosis. In both BCL-2 and BCL-xL, two central hydrophobic helices α5 and α6 are surrounded by five amphipathic α-helices. Both proteins contain a surface-exposed hydrophobic cleft, formed by BH (1–3) regions, which participates in hetero- and homodimerization among family members. Heterodimerization between anti- and pro-apoptotic members is critical for regulating the multiple death pathways influenced by this family of proteins (1, 5, 6, 22). In heterodimers, the hydrophobic binding cleft of the anti-apoptotic partner is occupied by the BH3 region of a pro-apoptotic member and, in cells, this interaction is typically triggered by upstream signaling. The r.m.s.d. 1 (root mean square deviation) values for backbone atoms of BCL-2 and BCL-xL is less than 2 Å, making them close homologues (17). Similarly, 2–3 Å r.m.s.d. was observed between BCL-2 (or BCL-xL) and BAX (17, 21). It was suggested that the loop structure connecting helices α1 and α2 in these proteins is largely disordered (15, 17). Solution structure analysis also revealed that there is more negative potential at the bottom of the binding site in BCL-xL than in BCL-2, which probably accounts for the higher affinity of BCL-xL for the BH3-peptides of BAD and BAK (17, 23). The helix α2 contains the BH3-region and appears to be packed closer to the hydrophobic pocket in BCL-2 and BCL-xL than in BAX (17, 21). The overall α-helical

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† To whom correspondence should be addressed: Dept. of Biochemistry, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec H3G 1Y6, Canada. Tel.: 514-398-7287; Fax: 514-398-7384; E-mail: kalle.gehring@mcgill.ca.

1 The abbreviations used are: r.m.s.d., root mean square deviation; RDC, residual dipolar coupling; HSQC, heteronuclear single-quantum correlation; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; SELDI-TOF, surface-enhanced laser desorption ionization-time of flight.
fold of BH3-only BID is similar to that of other BCL-2 family proteins but the r.m.s.d. values for backbone atoms are relatively larger (4 – 5 Å) (19 – 21). Finally, the three-dimensional structures of the BCL-2 family proteins show significant resemblance to bacterial pore-forming proteins such as diphtheria toxin and colicins (22, 24). Moreover, in vitro studies demonstrated that BCL-2, BCL-xL, and BAX form non-conducting channels in artificial membranes, an activity that might contribute to their function in vivo (25 – 27).

There has been little reported on the structure-function relationship of BCL-w, despite the fact that BCL-w provides pleiotropic resistance against multiple death stimuli (28), is up-regulated in colorectal cancer (14), and plays an essential role in spermatogenesis (29). Here, we report the NMR structure of this protein, which, in addition to confirming that BCL-w is closely related to other family members, has uncovered a novel conformation adopted by helix α8 compared with the corresponding region in BCL-2 and BCL-xL. Helix α8 in the native BCL-w is juxtaposed to the transmembrane segment and, intriguingly, in the structure of the cytosolic region reported here, folds into the BH3-binding pocket of the protein where it influences interactions of BCL-w with BH3 ligands.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The cDNAs of wild-type human BCL-w and a P117V mutant, both lacking the C-terminal transmembrane (Δ172–193) region, were subcloned in pET-29b+ plasmid which provides a C-terminal His tag LEHHHHHHH (Novagen). The amino acid sequence conducting channels in artificial membranes, an activity that regions 1 where it influences interactions of BCL-w with BH3 ligands. The amino acid sequence was prepared similarly. The proteins were expressed in Escherichia coli BL21(DE3). For NMR, cultures were grown in M9 media supplemented with 15N ammonium chloride and/or 13C-enriched glucose to produce uniformly 15N- or 15N-,13C-labeled proteins. Cells were grown at 37 °C and BCL-w synthesis induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. Soluble BCL-w protein was purified by one step by Ni2+–affinity chromatography. Typically 7 – 9 mg of BCL-w were obtained from 1 liter of cell culture. Mass spectral analysis (Sciex API MS system, Thornhill) showed that the N-terminal methionine residues were absent. NMR samples contained 0.7 – 1.4 mM BCL-w protein in 90% H2O/10% D2O, 20 mM sodium phosphate (pH 7.3), 0.5 mM EDTA, and 3 mM dithiothreitol.

Unlabeled BH3-peptides of human BID (IKKNIARHLAQVGS-DSDMRSI-CONH2) and of human BAK (TKGGQVRQLAIGG-DDINRRY-CONH2) were synthesized (BRI-NRC, Montreal) and used for NMR studies of the BCL-w/BID-BH3-peptide complex and for determining the binding constants to BCL-w and BCL-xL. NMR Spectroscopy—NMR spectra were acquired at 25 – 30 °C on Bruker DRX-500 and Varian INOVA-800 spectrometers. The following experiments were used for backbone and side chain 1H, 13C, and 15N resonance assignments: HNCACB, CBCA(CO)HN, HNCA, HNCO, HN(CA)CO,1H TOCSY-(CO)HN, 13C TOCSY-(CO)HN, and HCCH-TOCSY (32). Values of 3J/2/2 and 3J/2/3 coupling constants, and sequential and medium-range NOE patterns. The protein structure was refined using standard protocol in CNS Version 1.1 (39). The numbers of different types of constraints are present in Table I, and NH RDCs for terminal residues (1 – 8 and 170 – 179) and the flexible loop region (residues 27 – 40) were not included in structure calculations. Manually assigned NOE distance constraints were classified according to the peak intensities as strong (2.5 – 3.0 Å), medium (2.0 – 4.0 Å), or weak (2.5 – 5.0 Å). Hydrogen bonds were given bounds of 1.7 – 2.3 Å (H–O) and 2.7 – 3.3 Å (N–O).

The resulting NMR structures were evaluated with SSBYL (Tripos Inc.) and PROCHECK (40) software. Color figures were generated with MOLSCRIPT (41), RASTER-3D (42), and GRASP (43). The BCL-w
The structures were superposed based on all α-helical residues only.

| TABLE I |
|-------------------|
| Structural statistics for BCL-w (P117V) |

| Restraints for structure calculations: | 316 |
| Intraresidue NOEs | 452 |
| Medium and long range NOEs | 359 |
| Hydrogen bonds | 160 |
| ϕ and ψ backbone angles | 202 |
| NH residual dipolar couplings | 142 |

| Final energies (kcal/mol): | 379 ± 15 |
| Ebond | 16.5 ± 3.6 |
| Eangle | 89 ± 13 |
| Esuper | 12.4 ± 1.8 |
| Erepel | 221 ± 13 |
| Eelec | 15.9 ± 3.3 |
| Eisor | 2.0 ± 1.3 |
| Emain | 24.8 ± 5.7 |

(r.m.s.d.) from idealized geometry:
- Bond (Å) 0.0025 ± 0.0003 |
- Bond angles (°) 0.35 ± 0.03 |
- Improper torsions (°) 0.23 ± 0.02 |

(r.m.s.d.) for experimental restraints:
- Distances (Å) 0.013 ± 0.002 |
- Dihedral angles (°) 0.26 ± 0.11 |
- Residual dipolar couplings (r.m.s.d.) from average structure (Å²):
  - Backbone atoms (N, Cα, C') 1.6 ± 0.3 |
  - All heavy atoms | 0.14 ± 0.02 |

Q-value 0.14 ± 0.02 |

For residues 10-24 and 42-168.

atomic coordinates have been deposited in the Protein Data Bank under accession code 1MK3. Structural data for related proteins were taken from Protein Data Bank entries of 1BXL for BCL-xL and 1F16 for BAX. The pairwise coordinate r.m.s.d. comparisons between these proteins was obtained by the DALI program (44).

**Measurement of Binding Constants**—Binding of BH3-peptides from BAK and BID to BCL-w and BCL-xL was determined by SELDI-TOF mass spectroscopy (surface-enhanced laser desorption ionization-time of flight). Briefly, 1 μg of BCL-w or BCL-xL and varying concentrations of peptide were incubated in 25 μl of phosphate-buffered saline at room temperature for 1 h. The incubation mix was then adjusted to 0.5 M NaCl, 20 mM imidazole, 0.1% Triton X-100, 50 mM sodium phosphate (pH 7.2), and applied to the binding spots on a nickel-activated immobilized metal affinity chip-protein chip using a bio-processor (Ciphergen). After 1-h binding, the chip was washed three times with 0.5 M NaCl, 20 mM imidazole, 0.1% Triton X-100, 25 mM sodium phosphate (pH 7.2), and the chip read in the PDS-II (Ciphergen) mass spectrometer. Peak intensities from the peptide signal in three separate experiments were used for the calculation of binding affinities (Kd). Peak intensities were normalized for the total ion current by the Protein Chip software (Version 3.0, Ciphergen).

**RESULTS AND DISCUSSION**

**Resonance Assignments**—The 1H-15N HSQC spectrum of 15N-enriched BCL-w showed good signal dispersion, which is typical for α-helical proteins of BCL-2 family (Fig. 2a). Five tryptophan sidechain H1 signals were distinctly visible; the most downfield signal (δH of 10.84 ppm) was broadened at room temperature. Unfortunately, the HSQC spectrum of wild-type BCL-w also contained signals from a second minor conformer (−20%). This second BCL-w form could not be removed by purification, varying buffer conditions, or denaturation/refolding procedures. In addition, many signals of wild-type BCL-w residues were weak or broad, and these residues belonged mostly to the region between helices a5 and a6.

Since such effects could be related to cis-trans isomerization of proline residues, we decided to replace proline 117 in BCL-w with a valine residue, which is present at the homologous position in the BCL-xL sequence (Fig. 1). The improved HSQC spectrum of the P117V mutant of BCL-w is shown in Fig. 2b. The spectrum did not contain the signals of the minor form, and more than 95% of backbone amide resonances could be identified and assigned by triple-resonance NMR techniques. The P117V substitution is unlikely to affect the function of BCL-w because of its location in a turn, outside of the conserved BH-(1-3) regions. This substitution resulted in small 1H,15N chemical shift changes only for residues 111-125 (maximum ΔδH of 0.13 ppm for Gly-120), which are close to the P117V substitution. The positions of other amide signals coincided with signals of the main isomeric form of wild-type BCL-w, which suggests that this main conformer contains a trans conformation for proline 117. Despite the significant improvement of in the NMR spectrum of BCL-w (P117V), the most downfield tryptophan indole H1 resonance (assigned to residue Trp-167) was still broad in comparison with other H1 signals. This broadening does not appear to be due to proline 117 isomerization.

**Protein Structure and Region Mobility**—The structure of BCL-w (P117V) was calculated based on a total of 1631 NMR-derived restraints including 1H,15N RDC (Fig. 3). The set of best structures with lowest energy is presented in Fig. 4; structural statistics is shown in Table I. Except for terminal residues and the flexible loop region (residues 25-41), the mean r.m.s.d. from the average structure was 0.60 Å for backbone atoms. All ϕ and ψ angles fell within the most favored and allowed regions of Ramachandran space. A ribbon representation of the BCL-w structure closest to the ensemble average is presented in Fig. 5a. It contains 8 principal α-helices arranged in a fold similar to that of other BCL-2 family proteins. The pairwise Cα-atomic coordinate r.m.s.d. between entire BCL-w and BCL-xL structures is 2.8 Å (DALI Z factor = 7.8), and the same r.m.s.d. between BCL-w and BAX proteins is 3.5 Å (Z = 11.3). Exclusion of the flexible loop, and the C and N termini of BCL-w (i.e. comparison for BCL-w residues 10–24 and 42–168),
The most surprising feature of the BCL-w structure is the engagement of the C-terminal helix α8 in the hydrophobic cleft (Fig. 5a). This is different from the structures of the anti-apoptotic BCL-xL protein (Fig. 5c) where helix α8 was either absent or oriented into solution (15, 16). It is similar to the conformation of the C-terminal transmembrane helix in the pro-apoptotic BAX protein (Fig. 5b) (21). The aromatic side chain of Trp-167 is directed into a very restricted space between helices α4 and α5 of BCL-w (Fig. 5d), which may partially explain the downfield chemical shift of the H$_N$(Trp-167) resonance.

Further differences between these proteins are evident in the analysis of $^{15}$N{¹H} heteronuclear NOE measurements of backbone amide mobility (Fig. 3). This analysis shows that the α-helices in BCL-w are less mobile (with NOEs more than 0.6) than the loop regions (NOEs between 0.4 and 0.6) and terminal tails (NOEs less than 0.4). More notably, helix α8 is distinctly more mobile (NOEs $-0.6$–$0.7$) than the other α-helices. The BCL-w helix α8 also appears to be more mobile than the corresponding C-terminal transmembrane helix in BAX (21). This suggests that the affinity of this intra-molecular binding is weaker in BCL-w than in BAX. The relatively poor affinity of helix α8 for the BCL-w cleft may be due to the small number of hydrophobic residues in this helix as compared with the C-terminal helix of BAX (Figs. 1 and Fig. 5b). There are also three negatively charged Glu and three positively charged Arg residues in the helix α8 of BCL-w. For BAX, it was proposed that the C-terminal α-helix can prevent dimerization between partners in solution due to its very tight binding in the hydrophobic cleft (21). This does not appear to be the case for BCL-w as the helix α8 displays significant mobility in the backbone amides.

**BCL-w/BH3-Peptide Complexes**—To study the interaction of BH3 region peptides from pro-apoptotic proteins with BCL-w, we made 1:1 complexes of wild-type BCL-w with 20-mer BH3-peptides derived from human BID, BAK, BAX, BAD, BIK, or BOK proteins. The analysis of $^1$H–$^{15}$N HSQC spectra showed that all these peptides, except BOK, interact tightly with BCL-w. The best quality NMR spectra were obtained for the BCL-w/BID-BH3-peptide complex, which was the most soluble and where amount of the BCL-w minor cis-proline 117 conformer was less than 5%. The amide signals in the complex (excluding helix α3 and the region between α5 and α6) were assigned by triple-resonance NMR spectroscopy. General analysis showed that the positions of BCL-w α-helices in the complex with the BID peptide were the same as in wild-type BCL-w, but that the C-terminal helix was unfolded into solu-

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**TABLE II**

| Protein                  | $K_d$ (nM) | $K_d$ (nM) |
|--------------------------|------------|------------|
| BAK-BH3                  | 280 ± 86   | 302 ± 49   |
| BAX-BH3                  | 992 ± 97   | 1270 ± 31  |
| BCL-w (P117V) (Δα8)      | 84 ± 13    | 463 ± 84   |
| BCL-xL                   | 85 ± 13    | 448 ± 95   |

$^a$ Dissociation constants obtained using SELDI-TOF mass spectroscopy as the mean of three observations ± S.D.

$^b$ Deleted for amino acids 154-171.
tion. As evidence of this, measured values of 2J(1H,13CH) couplings for practically all the C-terminal residues 157–168 were larger than 5 Hz. Also, in the complex, the H1 signal of Trp-167 was not broadened and was strongly shifted upfield (ΔδH1 = 0.7 ppm).

More detailed analysis of BID peptide binding to BCL-w was carried out by comparison of chemical shifts for backbone amide signals in HSQC spectra. The BCL-w residues showing the largest chemical shift changes, (Δ31H) shift + (Δ15N) shift × 0.2)1/2 in ppm, upon binding of BID peptide, were Phe-53 (0.34), Glu-54 (0.74), Thr-55 (0.84) in helix α2, Ser-83 (0.36), Leu-86 (0.43) in helix α4, Thr-93 (0.35), Arg-95 (0.45) in helix α5, and Ala-155 (0.66), Leu-156 (0.55), Glu-157 (0.88), Glu-158 (0.73), Ala-159 (0.54), Leu-162 (0.35), Ala-168 (0.75), Ser-169 (0.51), Val-170 (0.68) in the C-terminal region (including helix α8). These data led us to conclude that the binding site of BID peptide in BCL-w is similar to that of BCL-xL (Fig. 5c) and that the BID peptide binds via the hydrophobic cleft of BCL-w. The data also suggest that the C-terminal helix α8 of BCL-w is displaced from the cleft upon ligand binding. We expect that the BCL-w/BID peptide interaction analysis by SELDI-TOF mass spectroscopy supports this view (Table II). The apparent affinities of BCL-xL and wild-type BCL-w were similar for both BID and BAK-peptides reflecting the similarity of the hydrophobic binding pockets of these two proteins. Deletion of helix α8 of BCL-w resulted in a significant increase in the binding affinity. As evidence of this, measured values of 31P couplings (Δδ)2/2 in ppm, upon binding of BID peptide, were Phe-53 (0.73), Ala-159 (0.54), Leu-162 (0.35), Ala-168 (0.75), Ser-169 (0.51), Val-170 (0.68) in the C-terminal region (including helix α8).

The BH3-peptide interaction analysis by SELDI-TOF mass spectroscopy supports this view (Table II). The apparent affinities of BCL-xL and wild-type BCL-w were similar for both BID and BAK-peptides reflecting the similarity of the hydrophobic binding pockets of these two proteins. Deletion of helix α8 of BCL-w resulted in a significant increase in the binding affinity. As evidence of this, measured values of 31P couplings (Δδ)2/2 in ppm, upon binding of BID peptide, were Phe-53 (0.73), Ala-159 (0.54), Leu-162 (0.35), Ala-168 (0.75), Ser-169 (0.51), Val-170 (0.68) in the C-terminal region (including helix α8). These data led us to conclude that the binding site of BID peptide in BCL-w is similar to that of BCL-xL (Fig. 5c) and that the BID peptide binds via the hydrophobic cleft of BCL-w. The data also suggest that the C-terminal helix α8 of BCL-w is displaced from the cleft upon ligand binding. We expect that the BCL-w/BID peptide structure is similar to that of the BCL-xL/BAK-peptide complex (16) shown in Fig. 5c. Upon addition of ligand, the peptide binds tightly in the hydrophobic cleft of BCL-w protein and displaces the C-terminal helix α8. Nevertheless, helix α8 may modulate or prevent the binding of lower affinity peptides and proteins as observed for the BOK BH3-peptide. The BH3-peptide interaction analysis by SELDI-TOF mass spectroscopy supports this view (Table II). The apparent affinities of BCL-xL and wild-type BCL-w were similar for both BID and BAK-peptides reflecting the similarity of the hydrophobic binding pockets of these two proteins. Deletion of helix α8 from BCL-w resulted in a significant increase in the binding affinities for BAK and BID BH3-peptides. This likely reflects increased accessibility of the binding cleft for interaction with the BH3-peptides. Surprisingly, the P117V substitution in BCL-w led to a 3–4-fold reduction in affinity for the BH3-peptides despite only minor effects in the NMR spectrum. We also investigated by NMR the consequences of deletion of residues 154–171 (including helix α8) from BCL-w and noted that this led to strong line broadening in 1H,13C HSQC spectra of BCL-w at 1 mM or higher protein concentrations. Thus the deletion of helix α8 appears to lead to weak homodimerization/oligomerization of BCL-w in solution.

In conclusion, our results suggest that BCL-w has a structure very similar to that of BCL-2 and BCL-xL, consistent with the ability of BCL-w to regulate apoptosis in response to diverse death signals. The ability of helix α8 to fold into the BH3-binding cleft of BCL-w suggests, however, an additional level of regulation that was not observed before in BCL-2 and BCL-xL. In this regard, BCL-w exhibits similarities to the soluble form of BAX in which the C-terminal transmembrane helix is sequestered within the peptide binding cleft on the protein surface and prevents heterodimerization with cytosolic proteins. Helix α8 of BCL-w might play a similar regulatory role, restricting interactions of BCL-w to only those partners that are capable of displacing helix α8 from the BH3-binding cleft.

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Solution Structure of Human BCL-w: MODULATION OF LIGAND BINDING BY THE C-TERMINAL HELIX
Alexei Yu. Denisov, Murthy S. R. Madiraju, Gang Chen, Abdelkrim Khadir, Pierre Beauparlant, Giorgio Attardo, Gordon C. Shore and Kalle Gehring

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