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Regions essential for the interaction between Bcl-2 and SMN, the spinal muscular atrophy disease gene product

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Abstract

The SMN gene is implicated in spinal muscular atrophy (SMA), and its product has been shown to interact with Bcl-2 protein to enhance its anti-apoptotic activity. In this study, we determined the regions that were essential for the interaction of Bcl-2 and SMN by co-immunoprecipitation of deletion mutants. Bcl-2 lacking its amino-terminal 20 amino acid residues or its carboxyl-terminal membrane-anchoring domain showed no or greatly reduced binding with SMN, respectively. However, Bcl-2 lacking other regions could still bind to SMN. Because Bcl-2 lacking the membrane-anchoring domain could bind to SMN in a yeast two-hybrid system, the amino-terminal region of Bcl-2 seems to be the most important domain for binding with SMN. A fragment of SMN encoded by exon 6 could bind to Bcl-2, but SMN lacking this region could not. From these results, we concluded that Bcl-2 and SMN proteins bound with each other at the amino-terminal region near the BH4 domain of Bcl-2 and the region encoded by exon 6 of SMN, both regions known to be important for their function. *Cell Death and Differentiation* (2000) **7,** 374–383.

Keywords: Bcl-2; SMN; spinal muscular atrophy; apoptosis

Abbreviations: BH, Bcl-2 homology; NMI, normal mouse IgG; NRS, normal rabbit serum; SMA, spinal muscular atrophy; SMN, survival motor neuron; VDAC, voltage-dependent anion channel

Introduction

Spinal muscular atrophy (SMA) is a common fatal autosomal recessive disorder characterized by degeneration of the anterior horn cells of the spinal cord, leading to progressive muscular atrophy.[1,2] The survival motor neuron (*SMN*) gene has been shown to be the determining gene for SMA.[3] A region of the *SMN* gene at chromosome 5q13 is duplicated as an inverted repeat so that there exists an additional centromeric copy gene (termed *^{c}BCD541* or *SMN^{c}*) which has five silent base substitutions and possesses the identical protein-coding capacity as the telomeric *SMN* gene. The 5q13 region also contains other genes, *NAIP*,[4] *BTF2p44*[5,6] and *H4F5*,[7] which are deleted in some SMA patients and might also be involved in the pathogenesis of SMA.

More than 95% of SMA patients show deletion of both telomeric *SMN* alleles or deletion of one allele with mutation in the other allele.[3,8–22] Consequently, only the centromeric copy of the *SMN* gene is functional in most SMA patients, and this produces both the full-length *SMN* transcript and a transcript lacking exon 5 or exon 7 by alternative splicing.[3] In contrast, the major product of the telomeric *SMN* gene is the full-length transcript.[3] A decrease of the *SMN* gene product[23] and/or changes in the ratio between full-length SMN and SMN lacking exons 5 or 7[3,24] may underlie the pathogenesis of SMA. The latter concept is supported by the observation that several mutations in the telomeric *SMN* gene of patients with severe SMA lead to alternative splicing that produces transcripts lacking exon 7.[3] On the other hand, because gene conversion of telomeric *SMN* to *SMN^{c}*,[25–27] resulting in increase of *SMN^{c}* gene copies, has been observed in some patients with mild SMA,[3,14,27,28] a higher number of copies of the *SMN^{c}* gene may reduce the severity of symptoms. However, other studies have not confirmed a correlation between *SMN^{c}* gene copy number and disease severity.[24,26,29,30]

Four independent processes have been proposed as the physiological roles of the *SMN* gene product: (1) SMN interacts with hnRNP U, with itself, and with fibrillarin, a small nucleolar RNA binding protein,[31] or with SIP1 and snRNP Sm core proteins (including B/B′ and D) during spliceosome synthesis,[32,33] and thus influences the efficiency of splicing;[34] (2) SMN interacts directly with RNA;[35,36] (3) SMN interacts with nuclear transcription activator E2 and thus influences E2-dependent transcriptional activation[37] and (4) SMN interacts with the anti-apoptotic protein Bcl-2 to synergistically enhance its anti-apoptotic activity.[38]

Bcl-2 is the first member of the still-growing Bcl-2 family, and this family is characterized by the conservation of Bcl-2 homology (BH) domains.[39,40] The Bcl-2 family consists of pro-apoptotic molecules, such as Bax, Bak, Bik, Bad, and Bid, as well as anti-apoptotic molecules, such as Bcl-2, Bcl-x_L, and Bcl-w.[39,40] Some pro-apoptotic Bcl-2 family proteins contain BH1, 2, 3 domains which are important for homodimerization and heterodimerization.[39,40] BH3 domain-only proteins, containing BH3 but not BH1 and BH2, as well as BH3 oligopeptide induce apoptosis, suggesting

that the BH3 domain is responsible for pro-apoptotic activity of this family.[39,40] In most anti-apoptotic Bcl-2 family proteins, the BH4 domain at the amino terminal region is conserved in addition to the BH1, 2, and 3 domains, and it seems important for preventing apoptosis.[39,40] A loop domain was also found by structural analysis of human Bcl-x_L protein,[41] and it is thought to be involved in modulation of the activity of the anti-apoptotic family members, probably through phosphorylation.[42,43] These proteins are partly regulated through interactions between selected pairs of pro-apoptotic and anti-apoptotic family members,[44] by binding of other proteins like Bag-1[45] and SMN,[38] or by chemical modifications including phosphorylation by Raf-1,[46] Akt,[47,48] PKA,[49] or PKC.[50]

Several functions of Bcl-2 have been proposed, such as prevention of apoptotic mitochondrial changes including cytochrome c release and membrane potential loss[51–53] by closing the voltage-dependent anion channel (VDAC),[54] or binding and sequestering Apaf-1, a caspase-activating protein.[55–59]

We have previously shown that synergistic anti-apoptotic activity is not observed between Bcl-2 and SMN,^{Y272C} a mutant found in some SMA patients, and that SMN/Bcl-2 synergism was cancelled by co-expression of SMN lacking the C-terminal region encoded by exon 7 (called SMNIa).[38] Thus, the dominant negative activity of SMNIa, whose proportion in all SMN products shows an increase in SMA patients, might play an important role in the pathogenesis of SMA. Because Bcl-2 expression in lower motor neurons is reduced after birth in both mice[60] and humans,[61] promotion of the anti-apoptotic activity of Bcl-2 by SMN might be required for lower motor neuron survival.

In the present study, we determined the essential regions for the binding of Bcl-2 with SMN as the first step towards understanding the mechanisms of their synergistic anti-apoptotic activity. We found that Bcl-2 and SMN bound to each other through the amino-terminal region near the BH4 domain of Bcl-2 and the region encoded by exon 6 of SMN.

Results

Essential region of Bcl-2 for binding to SMN

To understand the molecular mechanisms of the synergistic anti-apoptotic effect of human Bcl-2 (hBcl-2) and human SMN, we analyzed the interaction of these proteins. Haemagglutinin (HA)-tagged SMN protein (HA-SMN) was expressed together with hBcl-2 protein by transient co-transfection of COS-7 cells with two plasmids (pCAGGS-HA-SMN and pCAGGS-hbcl-2). After immunoprecipitation of cell lysates, the product was examined by Western blot analysis. When both HA-SMN and hBcl-2 were expressed, anti-HA antiserum, but not control antiserum, caused co-immunoprecipitation of hBcl-2 with HA-SMN (Figure 1A). Conversely, an anti-human Bcl-2 polyclonal antibody caused co-immunoprecipitation of HA-SMN with hBcl-2 (data not shown). On the other hand, co-immunoprecipitation of hBcl-2 was not detected when hBcl-2 was expressed without SMN (Figure 1A). These results indicated that SMN and hBcl-2 could interact with each other in mammalian cells, confirming our previous observations.[38]

When chicken Bcl-2 or human Bcl-x_L, homologues of human Bcl-2, were expressed with HA-SMN, neither of these proteins was co-immunoprecipitated with HA-SMN (Figure 1B,C). These findings suggested that human SMN binds specifically to human Bcl-2.

To determine the regions of human Bcl-2 that were necessary for specific binding to SMN, we constructed various human *bcl-2* mutant genes. A series of deletion mutants were constructed as described in Materials and

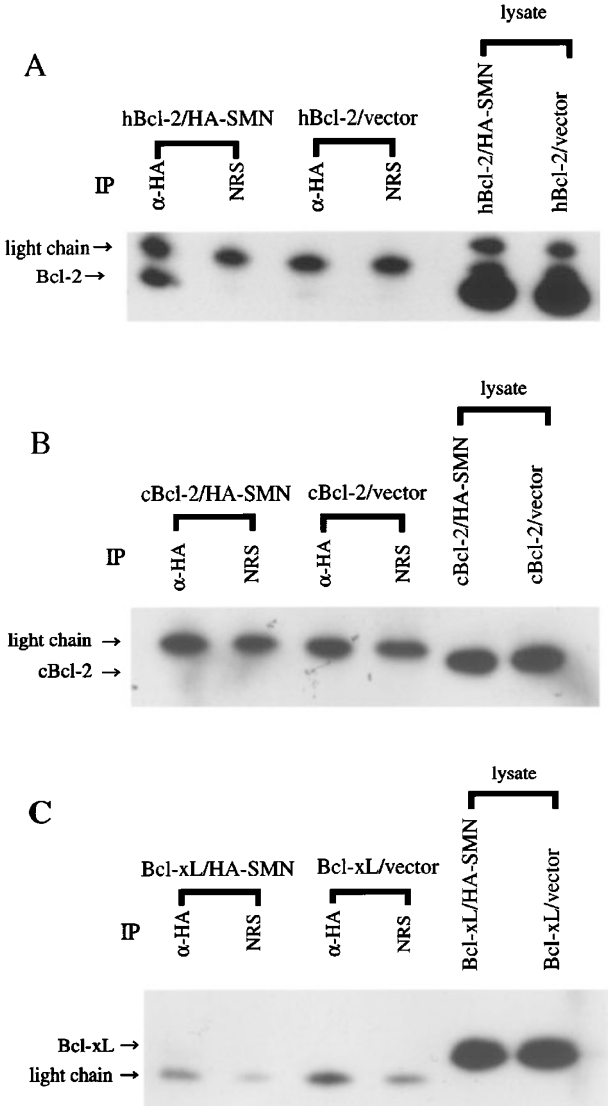

Figure 1 Co-immunoprecipitation of hBcl-2 and SMN. COS-7 cells were transfected with the indicated plasmids, and cell lysates were subjected to immunoprecipitation (IP) using anti-HA rabbit antiserum (α-HA) and normal rabbit serum (NRS) as the control, as described in Materials and Methods. After gel electrophoresis, hBcl-2 (**A**), cBcl-2 (**B**) and hBcl-x_L (**C**) proteins in immunoprecipitates were detected by Western blotting using an anti-hBcl-2 monoclonal antibody (**A**), an anti-cBcl-2 mouse monoclonal antibody (**B**), and an anti-hBcl-x_L mouse monoclonal antibody (**C**), respectively. Part of each precleared lysate obtained before immunoprecipitation was also loaded (lysate)

Methods, and are shown in Figure 2. Δ1, Δ2, Δ5, Δ6, Δ8, Δ9, Δ10 and Δ11 each lacked 20 amino acid residues, and only Δ7 lacked 24 amino acid residues (Figure 2). Two mutants, Δloop and Δ12, were also constructed without the loop domain and the membrane-anchoring domain of hBcl-2, respectively (Figure 2). Each mutant gene was subcloned into the pUC-CAGGS expression plasmid.

Using these deletion mutants of *bcl-2*, we examined the regions of hBcl-2 that were essential for binding to SMN by immunoprecipitation. We first examined the interaction of SMN with hBcl-2 lacking the regions mostly covering the BH1, 2, and 3 domains and loop domain. When hBcl-2 Δloop, Δ5, Δ8, and Δ10 mutants were expressed together with SMN in COS-7 cells, anti-HA antiserum co-immunoprecipitated these hBcl-2 proteins with HA-SMN to a similar extent as occurred with the full-length hBcl-2 protein (Figure 3A), indicating that the BH1, 2, 3 domains and the loop domain were not required for binding of hBcl-2 to SMN. Similarly, hBcl-2 Δ2, Δ6, Δ7, Δ9 and Δ11 proteins were co-immunoprecipitated with HA-SMN by anti-HA antiserum (Figure 3B), indicating that the regions removed in these proteins were also non-essential for the interaction of hBcl-2 with SMN.

In contrast, deletion of the amino terminal 20 amino acid residues near the BH4 domain of hBcl-2 (hBcl-2 Δ1) completely abolished the interaction of hBcl-2 with HA-SMN (Figure 3C). Consistently, an anti-human Bcl-2 polyclonal antibody did not co-immunoprecipitate HA-SMN with hBcl-2 Δ1 protein (data not shown). Deletion of the membrane-anchoring domain of hBcl-2 (hBcl-2 Δ12) greatly reduced the interaction with SMN (Figure 3D). From these results, we concluded that two domains near the BH4 and membrane-anchoring domains were important for the binding of hBcl-2 with SMN.

Essential region of SMN for binding to hBcl-2

To determine the region of SMN that was essential for interaction with hBcl-2, we constructed the following exon-based deletion mutants of *SMN*: SMNexon1234, SMNexon567, SMNexon56, SMNexon5, and SMNexon6 (Figure 4). All of the mutants were HA-tagged and subcloned into the pUC-CAGGS expression vector.

We then analyzed the interaction of the products of these *SMN* deletion mutants with hBcl-2 by immunoprecipitation. As shown in Figure 5A, hBcl-2 was not co-immunoprecipitated with SMNexon1234. On the other hand, hBcl-2 was detected in the immunoprecipitates by anti-HA antiserum when expressed together with SMNexon567 and exon56 (Figure 5B), suggesting that the region coded by exons 5 and 6 of the *SMN* gene was responsible for binding to hBcl-2. To determine whether exons 5 or 6 alone was sufficient for binding, we next examined the interaction of SMNexon5 and SMNexon6 with hBcl-2. As shown in Figure 5C, hBcl-2 was co-immunoprecipitated with SMNexon6 by anti-HA serum, but

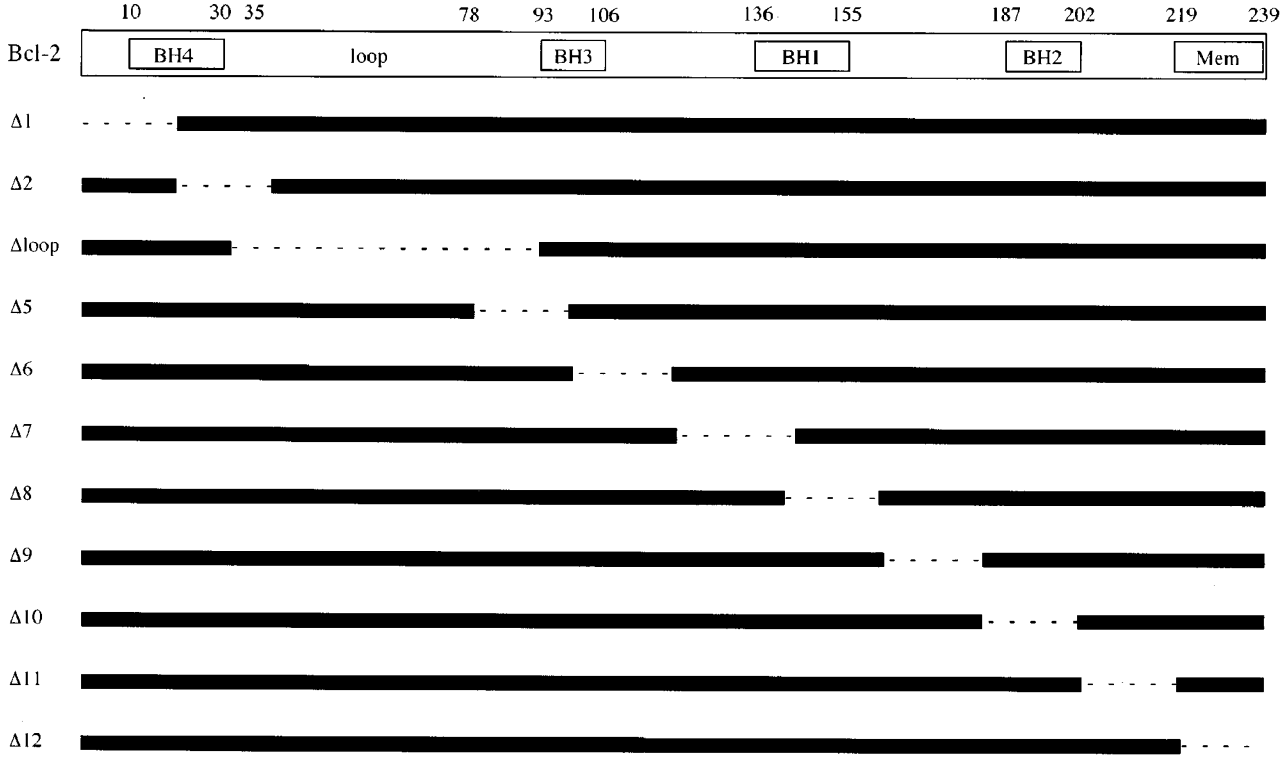

Figure 2 Diagram of hBcl-2 deletion mutants. The deleted and retained regions are shown by dotted lines and thick bars, respectively. The positions of BH1, BH2, BH3, and BH4, the loop domain, and the membrane-anchoring domain (Mem) are shown in the open box at the top of the figure. Numbers indicate the amino acid residues from the N-terminus. Most of BH1, BH2, and BH3 or the membrane-anchoring domain is lost in Δ8, Δ10, Δ5, and Δ12, respectively. The N-terminal half of BH4 and the entire loop domain are deleted in Δ1 and Δloop, respectively

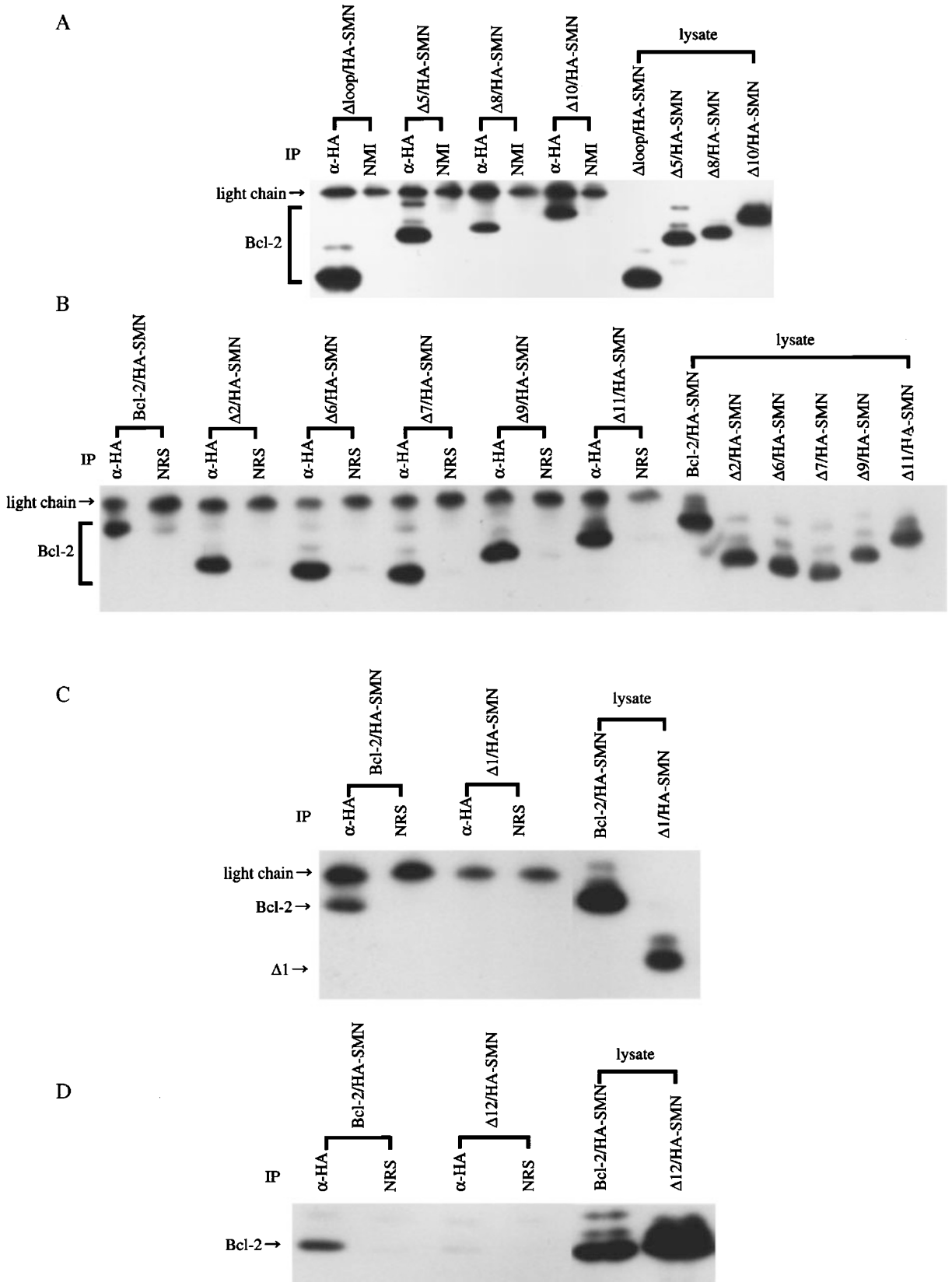

Figure 3 Importance of the N-terminal region and the membrane-anchoring domain for binding of hBcl-2 with SMN. (**A**) COS-7 cells were transfected with the indicated plasmids, and the cell lysates were subjected to immunoprecipitation using an anti-HA monoclonal antibody and normal mouse IgG (NMI) as the control. After gel electrophoresis, hBcl-2 proteins with the respective deletions were detected by an anti-hBcl-2 polyclonal antibody. (**B – D**) COS-7 cells were transfected with the indicated plasmids, and cell lysates were subjected to immunoprecipitation using anti-HA rabbit antiserum and normal rabbit serum (NRS) as the control. After gel electrophoresis, hBcl-2 proteins were detected by an anti-hBcl-2

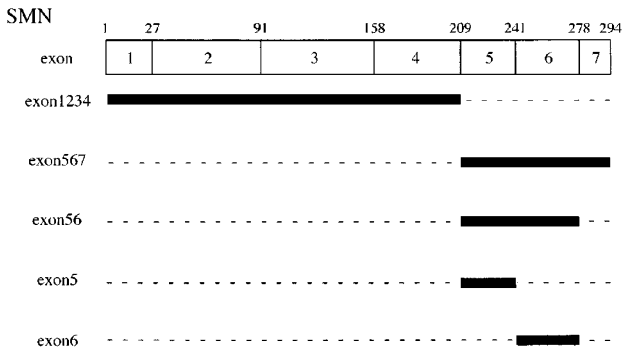

Figure 4 Diagram of SMN deletion mutants. Positions corresponding to each exon are shown in the open box and numbers indicate amino acid residues from the N-terminus. Deletion mutant proteins lacked the dotted regions and retained the regions shown by thick bars

not with SMNexons. 5. These results indicate that SMN interacts with hBcl-2 through the region encoded by exon 6.

Discussion

The SMN gene is implicated in the pathogenesis of SMA. Several actions of the SMN gene product have been proposed to contribute to SMA, such as involvement in splicing[34] and enhancement of the anti-apoptotic activity of hBcl-2 protein.[38] In the present study, we determined the essential regions for the interaction of hBcl-2 and SMN using deletion mutants. We found that hBcl-2 lacking the amino-terminal 20 amino acid residues did not show any detectable binding to SMN, while hBcl-2 lacking the membrane-anchoring domain (hBcl-2 Δ12) showed greatly reduced binding. Because hBcl-2 Δ12 bound to SMN in a yeast two-hybrid system,[38] these results strongly suggest that hBcl-2 binds to SMN through the amino-terminal region near BH4. The membrane-anchoring domain of hBcl-2 is required for its localization to the membrane, and hBcl-2 lacking the membrane-anchoring domain moves to the cytoplasm.[62] Therefore, the greatly reduced binding between SMN and hBcl-2 Δ12 might have been due to its change in subcellular localization, since there is little SMN in the cytoplasm.[38] We also found that the region encoded by exon 6 of SMN bound to hBcl-2, and SMN lacking this region did not, suggesting that the region encoded by exon 6 was responsible for binding to hBcl-2. From these results, we concluded that hBcl-2 and SMN bind to each other through the amino-terminal region of hBcl-2 near BH4 and the region of SMN encoded by exon 6.

A comparison of the amino acid sequences of the amino terminal regions of hBcl-2, cBcl-2 and hBcl-x_L is shown in Figure 6. BH4 spans the regions deleted in hBcl-2 Δ1 and Δ2. Because hBcl-2 Δ2 binds with SMN, the C-terminal half of BH4 should not be involved in this interaction. Although the N-terminal half of BH4, corresponding to the C-terminal half of the region deleted in hBcl-2 Δ1, contains some substitutions of amino acid residues between hBcl-2 and hBcl-x_L, the substituted residues have a similar hydrophobicity, so that the region is well conserved among these proteins. However, the N-terminal half of the region deleted in hBcl-2 Δ1 shows marked differences between hBcl-2 and hBcl-x_L, especially hBcl-2 having extra six amino acid residues. Because cBcl-2 did not bind to SMN, the difference of the amino acid residues in these regions of hBcl-2 and cBcl-2 might be responsible for the SMN-binding ability. These observations suggest that SMN might recognize the N-terminal half of the region deleted in hBcl-2 Δ1.

Figure 7 shows a comparison of the 3D-structures of hBcl-x_L and hBcl-2. Based on the published structures of human Bcl-x_L[41] and rat Bcl-x_L,[63] the structure of hBcl-2 was predicted by computer modeling. Because no information was available on the N-terminal six amino acid residues and the loop regions of hBcl-2, the positions of the residues in these regions are not displayed. The overall structures of both proteins are similar, but some critical differences can be seen around BH4. The N-terminal α-helix (α1) of hBcl-2 is shorter than that of hBcl-x_L, and the distribution of acidic and basic amino acid residues in α1 differs between hBcl-2 and hBcl-x_L. The α1 region almost covers BH4, which is conserved in many anti-apoptotic Bcl-2 family proteins and is important for the prevention of apoptosis.[64–68] Several biochemical functions of BH4 region have been proposed. BH4 region of hBcl-x_L was reported to be required for interaction with Ced-4 (a *C. elegans* homolog of Apaf-1) to form apoptosome.[68] We have recently shown that the BH4 oligopeptides of hBcl-x_L and hBcl-2 could shut off the voltage-dependent anion channel (VDAC) in the same way as the full-length proteins in a reconstituted VDAC-liposome system and these peptides can also prevent apoptotic changes in isolated mitochondria and apoptotic cell death.[69] Binding of SMN to the N-terminal half of the region deleted in hBcl-2 Δ1 might induce conformational changes in BH4. Because α1 is laid on the body of hBcl-2, conformational changes of BH4 might expose this region, and the exposure of BH4 by SMN might increase the anti-apoptotic activity of hBcl-2 possibly by increasing the affinity to VDAC and/or Apaf-1/Ced-4. Alternatively, the possible conformational changes induced by binding of SMN might stabilize the α-helix (α1) of hBcl-2, and may mediate the full anti-apoptotic activity of hBcl-2. Although we failed to detect any interaction between SMN and hBcl-2 peptides containing the region deleted in hBcl-2 Δ1 and BH4 (unpublished observations), this may have been caused by the conformational differences of α1 in solution relative to that on the protein. To elucidate the reason for the enhanced anti-apoptotic activity of Bcl-2 bound to SMN, structural analysis of the hBcl-2/SMN complex is probably necessary.

The region encoded by exon 6 is the most highly conserved region of SMN protein from yeast and *C. elegans* through mammals.[18,36,70] This region was reported to be responsible for binding to Sm core proteins,[32] self-association,[71] and binding to transcription activator E2.[37] Analysis of missense mutations of the *SMN* gene in SMA patients with heterozygous telomeric *SMN* gene deletion has shown that these mutations cluster in exon 6.[3,18,19,72–74] Although several mutations have been found in other regions, most of these are frame-shift mutations or

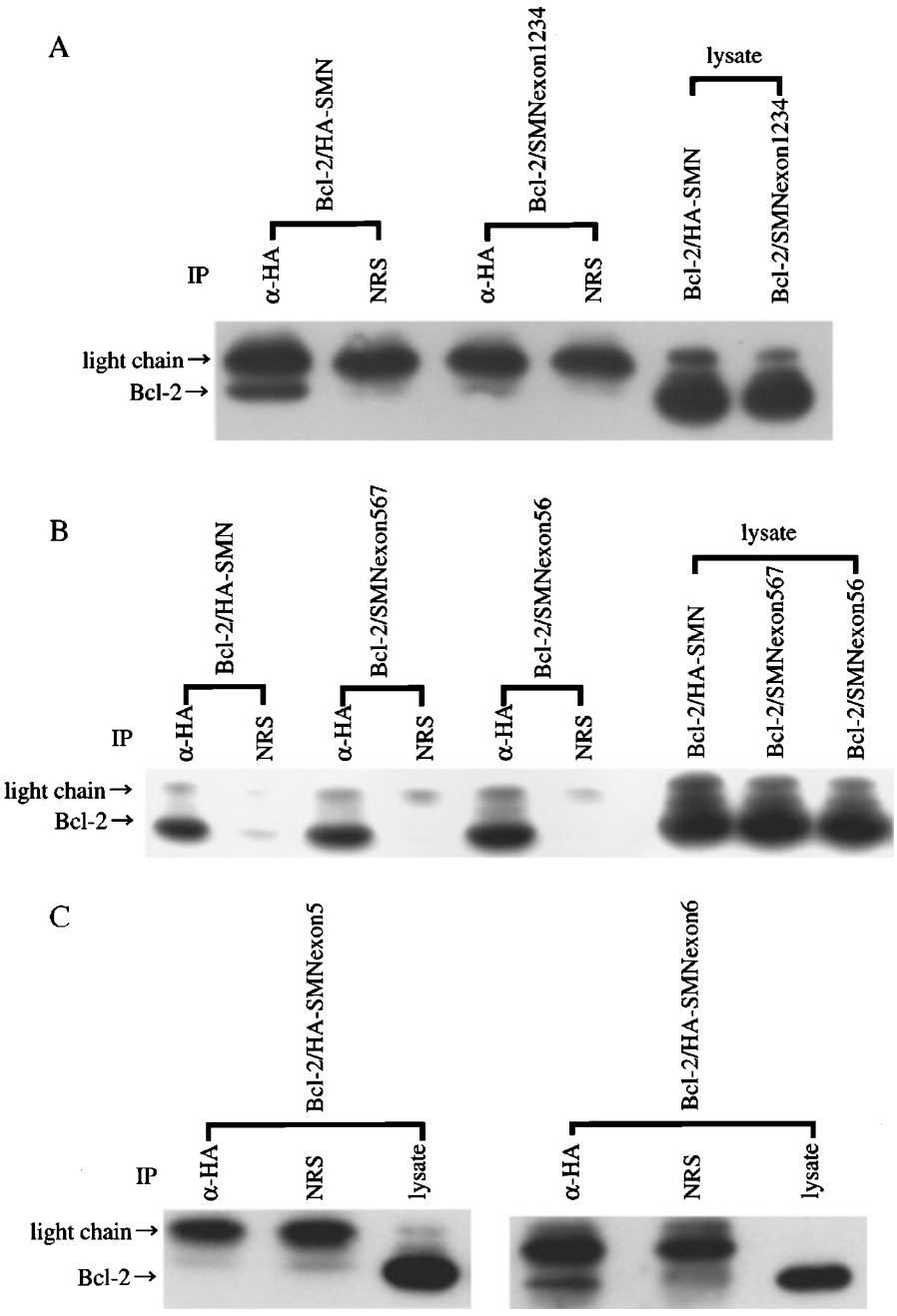

Figure 5 Exon 6 of SMN is responsible for binding to hBcl-2. (**A – C**) COS-7 cells were transfected with the indicated plasmids, and cell lysates were subjected to immunoprecipitation using anti-HA rabbit antiserum and normal rabbit serum (NRS) as the control. After gel electrophoresis, hBcl-2 was detected using an anti-hBcl-2 monoclonal antibody

nonsense mutations, so that SMN protein containing exon 6 was not produced by these mutants. These observations strongly suggest that the region encoded by exon 6 is the most important domain of SMN protein to exert its function. Actually, SMN protein with amino acid substitution $Y^{272}C$ in the exon 6 region, which has been found in some SMA patients, does not show any synergism with Bcl-2.[38] We speculate that the lack of synergistic effect with Bcl-2 of the SMNIa mutant lacking exon 7 is due to the conformational change of SMN protein by loss of the region. Alternatively, the region encoded by exon 6 may serve as the binding site for Bcl-2, and the functional site may reside in the region encoded by exon 7. Taken together with the fact that the exon 6 region of the *SMN* gene binds to hBcl-2 protein, the binding of SMN to Bcl-2 at this region seems likely to ensure motor neuron survival. Loss of the interaction and the synergistic anti-apoptotic activity seems to underlie the pathogenesis of SMA.

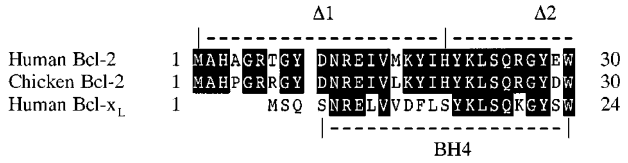

Figure 6 Alignment of the amino acid sequences of the N-terminal regions of hBcl-2, cBcl-2, and hBcl-x_L. The amino acid sequences of the N-terminal regions of hBcl-2, cBcl-2, and hBcl-x_L are shown. Regions corresponding to BH4 and removed in hBcl-2 Δ1 and Δ2 are indicated. Residues conserved in more than two proteins are highlighted

Materials and Methods

Plasmids

pCAGGS-hbcl-2 and pCAGGS-HA-SMN were described previously.[38] hBcl-2 deletion mutants were constructed by using *Hind*III linker to join upstream and downstream fragments obtained by polymerase chain reaction (PCR). The hBcl-2Δ1, Δ2, Δloop, Δ5, Δ6, Δ7, Δ8, Δ9, Δ10, Δ11, and Δ12 genes lacked the regions coding for a.a. 2–21, 22–41, 31–92, 81–100, 102–121, 122–145, 142–161, 162–181, 182–201, 202–221, 222–239 of hBcl-2, respectively. SMN deletion mutants were also constructed by PCR with the nine codons of the

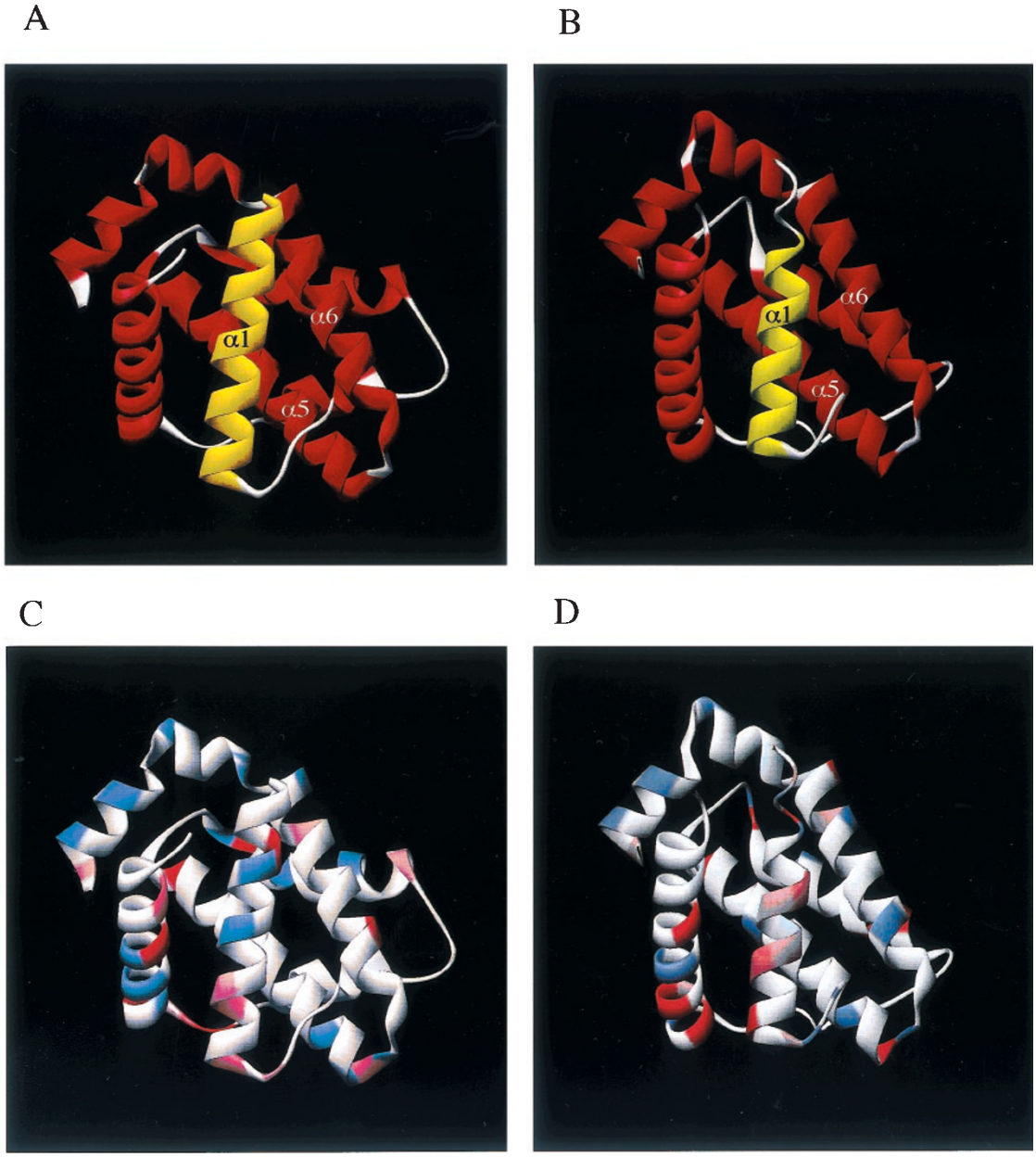

Figure 7 Comparison of the 3D-structure of hBcl-x_L and hBcl-2. Ribbon models of hBcl-x_L (**A** and **C**) and hBcl-2 (**B** and **D**) are shown. The X-ray structure of hBcl-x_L (entry 1MAZ in the Brookhaven database) was depicted using WebLab ViewerLite, and the structure of hBcl-2 was generated as described in Materials and Methods. The 1st to 27th and 81st to 196th residues of hBcl-x_L are shown, as are the 6th to 32nd and 87th to 203rd residues of hBcl-2. The first α-helix (α1) is colored yellow and the positions of α5 and α6 are shown in **A** and **B**. In **C** and **D**, acidic and basic residues are shown in blue and red, respectively

HA-epitope[75] being fused to the N-terminal codon of each gene. SMNexon1234, SMNexon567, SMNexon56, SMNexon5 and SMNexon6 contained the regions coding for a.a.1–209, 210–294, 210–278, 210–241, 242–278 of SMN, respectively. Each mutant gene was sequenced and integrated into the pUC-CAGGS expression vector[76] bearing a β-actin promoter and CMV enhancer.

Antibodies

The antibodies used were as follows: anti-hBcl-2 hamster monoclonal antibody (Pharmingen; clone 6C8), anti-hBcl-2 rabbit polyclonal antibody (α-hBcl-2),[77] anti-hBcl-x_L mouse monoclonal antibody (Transduction; clone 44), anti-cBcl-2 mouse monoclonal antibody (prepared for this study), anti-HA tag rabbit antiserum (α-HA: Medical and Biological Laboratories, Japan), and anti-HA tag mouse monoclonal antibody (Boehringer Mannheim; clone 12CA5). Normal rabbit serum (NRS: Medical and Biological Laboratories, Japan), normal mouse IgG2b (NMI: Bethyl), and normal rabbit IgG (Medical and Biological Laboratories, Japan) were used as control antibodies for anti-HA tag rabbit antiserum, 12CA5, and the other rabbit polyclonal antibodies, respectively.

Transfection and immunoprecipitation

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM) and 0.45% glucose at 37°C under humidified 10% CO_2. The indicated combinations of pCAGGS-hbcl-2, pCAGGS-HA-SMN, and their mutant constructs were transfected into COS-7 cells (at 50% confluence in 10-cm dishes), using LipofectAmine (Gibco) and 5 μg of each plasmid. In some cases, pCAGGS-cbcl-2 or pCAGGS-hbcl-x_L was transfected together with pCAGGS-HA-SMN. Cells were incubated for 36–42 h in DMEM supplemented with 10% FBS and were harvested for immunoprecipitation. The following procedures were carried out at 4°C. After washing with PBS, cells were lysed by sonication in lysis buffer (10 mM HEPES, pH 7.5, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% NP-40, 0.1 mM p-APMSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Cell debris were removed by centrifugation at 15 000 $\times$ *g* for 15 min. Lysates were precleared with control antibodies and 5% (v/v) protein G-sepharose (Pharmacia) for 60 min, and were subsequently incubated with specific antibodies for 90 min. Immunoprecipitates were captured with 5% (v/v) protein G-sepharose for 60 min, and were washed five times with lysis buffer. Immunoprecipitates from 10^5 cells were solubilized in SDS–PAGE sample buffer, subjected to SDS–PAGE on 12.5% gels and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore). Part of each precleared lysate obtained before immunoprecipitation was also loaded to show equivalence of the amount of proteins in lysates. The filters were incubated with specific antibodies and the bound antibody was visualized using horseradish peroxidase-conjugated specific secondary antibodies combined with a chemiluminescence enhancer (ECL, Amersham).

Computer modeling of Bcl-2

The structure of human Bcl-2 was modeled by ProModII[78] on a SWISS-MODEL Automated Protein Modeling Server. Modeling was based upon the coordinates of the X-ray structures of human Bcl-x_L,[41] and rat Bcl-x_L,[63] as well as NMR minimized average structures of human Bcl-x_L[41] and human Bcl-x_L/Bak peptide complex,[79] obtained from the Brookhaven database (accession Nos. 1MAZ, 1AF3, 1LXL, and 1BXL, respectively). Energy minimization was carried out using GROMOS96[80] with IFP43B1 as a parameter set, involving 200 cycles of steepest descent to satisfy 25/C-Factors and 300 cycles of conjugate gradient to satisfy 2500/C-Factors.

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