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Structural characterization of the BH3-like motif of hepatitis B virus X protein

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ABSTRACT

Hepatitis B virus X protein (HBx) is a multifunctional protein, which is considered to be an essential molecule for viral replication and the development of liver diseases. Recently, it has been demonstrated that HBx can directly interact with Bcl-2 and Bcl-x_L through a sequence (termed the BH3-like motif) that is related to the BH3 motif of pro-apoptotic BH3-only proteins. Here, we present the first structural characterization of the HBx BH3-like motif by circular dichroism and NMR spectroscopies. Our results demonstrated that the HBx BH3-like motif has the ability to form an α-helix, and the potential helical region involves residues L108–L134. This is a common characteristic among the BH3 peptides of pro-apoptotic BH3-only proteins, implying that HBx may interact with Bcl-2 and Bcl-x_L, by forming an α-helix, similar to the interaction mode of other BH3 peptides with Bcl-2 and Bcl-x_L.

1. Introduction

Chronic hepatitis B virus (HBV) infection is one of the major causes of the development of liver diseases including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [1,2]. HBV belongs to the *Hepadnaviridae* family of viruses and its genome contains four overlapping open reading frames that encode four major viral proteins: core protein, surface protein, polymerase and X protein (HBx). Among them, HBx has been closely associated with the development of HCC, according to the following evidence: (i) HBx alone promotes liver tumorgenesis in transgenic mice [3–5]. (ii) HBx and the C-terminal truncated HBx are integrated into host cell DNA found in patients with HCC [2,6,7]. However, the detailed mechanism on HCC development by HBx is not fully understood.

HBx (154 residues) possesses several domains or motifs, such as a transactivation domain [8], a DNA damage-binding protein 1 (DDB1) binding motif [9], a p53-binding domain [10–12] and an anti-apoptotic protein-binding motif (termed a BH3-like motif) [13,14]. HBx interacts with many proteins, and regulates cellular transcription, proliferation, DNA repair and apoptotic cell death, via its domains or motifs [15,16]. These functions are well correlated with HBV replication and the development of HCC. Recently, it has been demonstrated that HBx can directly interact with anti-apoptotic proteins, Bcl-2 and Bcl-x_L, through its BH3-like motif, which results in elevated cytosolic calcium, efficient viral DNA replication and the induction of programmed cell death [13,14].

The Bcl-2 family of proteins is important regulators of apoptotic cell death and is divided into two groups, anti- and pro-apoptotic proteins [17]. Anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, inhibit apoptosis and mainly contain four Bcl-2 homology (BH) domains (BH1–BH4). Pro-apoptotic proteins, such as Bak and Bax, promote apoptosis and contain three BH domains (BH1–BH3), whereas those with a single BH domain, referred to as BH3-only proteins (e.g. Bim, Bad and Bmf), interact with anti-apoptotic proteins, resulting in the inactivation of anti-apoptotic proteins. The opposing function of this family of proteins regulates cell survival or death by a balance between them.

Many structural studies of the BH3 peptide–Bcl-x_L complex have revealed that the BH3 peptide forms an amphipathic α-helix and the hydrophobic residues in BH3 peptides fit into a hydrophobic groove of Bcl-x_L [18–21], whereas the BH3 peptide alone is intrinsically unstructured [22]. Furthermore, it has been demonstrated that the helix propensity of the Bad BH3 peptide is essential

Abbreviations: Bcl-2, B-cell lymphoma 2; BH, Bcl-2 homology; CBB, Coomassie brilliant blue; CD, circular dichroism; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; His_{10}-Ub-HBx(101–136), decahistidine-tagged ubiquitin fusion of HBx(101–136); HSQC, heteronuclear single quantum coherence.

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for the interaction with Bcl-x_L, because increases in helical content in the presence of TFE correlate with an increase in the binding affinity to Bcl-x_L [19]. However, it is unclear whether the HBx BH3-like motif has an α-helix structure or the intrinsic propensity to form an α-helix. Therefore, we focus on the structural characterization of the HBx BH3-like motif as the first attempt to understand the interaction between HBx and anti-apoptotic proteins, Bcl-x_L and Bcl-2.

In this study, we examined the structural feature of residues 101–136 of HBx, which contains the BH3-like motif, and is termed HBx(101–136), using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopies. Our results showed that HBx(101–136) is a random coil in aqueous solution, whereas residues L108–L134 of HBx(101–136) adopts an α-helix structure in the presence of 2,2,2-trifluoroethanol (TFE). Our results are in good agreement with those of other BH3 peptides reported previously [18,19,23], suggesting that HBx may interact with its target molecules, Bcl-2 and Bcl-x_L, by forming an α-helix structure.

2. Materials and methods

2.1. Sample preparation

HBx(101–136) was expressed in *Escherichia coli* strain BL21(DE3) or BL21(DE3) pLysS, using a ubiquitin fusion protein system [24,25]. HBx(101–136) was fused to the C-terminus of ubiquitin with a decahistidine-tag at the N-terminus and the ubiquitin fusion protein is referred as to His_{10}-Ub-HBx(101–136) in this article. LB media were used to prepare the non-labeled HBx(101–136). M9 minimal media containing $^{15}NH_4Cl$ or $^{15}NH_4Cl/^{13}C_6$-D-glucose as the sole nitrogen and carbon sources were used to prepare uniformly ^{15}N-labeled HBx(101–136) or $^{13}C/^{15}$N-labeled HBx(101–136), respectively.

His_{10}-Ub-HBx(101–136) was solubilized in 6 M urea or 6 M guanidine hydrochloride (GdmCl) containing buffer A [50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 1 or 5 mM dithiothreitol (DTT), 10 mM imidazole], and the supernatant containing the denatured His_{10}-Ub-HBx(101–136) was obtained by centrifugation. The supernatant was loaded onto a His-accept column (Nacalai tesque Inc., Kyoto, Japan) and His_{10}-Ub-HBx(101–136) was refolded on the column by serially decreasing the concentration of urea or GdmCl from 6 M to 0 M. The refolded ubiquitin fusion protein was eluted with buffer A supplemented with 0.4 M imidazole and subsequently dialyzed against buffer B [50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM DTT]. His_{10}-Ub-HBx(101–136) was cleaved with yeast ubiquitin hydrolase at 37 °C. The resulting sample containing HBx(101–136) was passed through a His-accept column. The resulting sample containing HBx(101–136) was loaded onto a Sep-Pak Plus C_{18} Cartridge (Waters, MA, USA) and was eluted with approximately 50% acetonitrile and 0.1% TFA. The sample was dissolved in buffer C [50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM DTT] and 1 N NaOH was added to the sample to adjust the pH between 8.0 and 8.5. HBx(101–136) was finally purified with a Resource Q column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.2. CD spectroscopy

CD spectra were measured on a JASCO-820 spectropolarimeter at 25 °C with a 1-mm path length cuvette. CD data were recorded from 260 to 195 nm using 0.03 mM HBx(101–136) in 20 mM potassium phosphate (pH 6.8) and 1 mM DTT and with increasing concentrations of TFE from 0% to 50%. All data were obtained by the subtraction of a blank corresponding to the buffer. Mean residue molar ellipticity was calculated according to the peptide concentration, the number of residues in the peptide and the cell-path length.

2.3. NMR Spectroscopy

The NMR samples contained approximately 0.4 mM ^{15}N-labeled or 0.3 mM $^{13}C/^{15}$N-labeled HBx(101–136) in 20 mM potassium phosphate (pH 6.8), 20 mM NaCl, 2 mM DTT, 50% TFE-d_3, 0.5 mM DSS and 10% D_2O. NMR spectra were acquired at 25 °C using a Bruker AVANCE III 800 spectrometer. Backbone ^{1}H, ^{13}C, and ^{15}N resonance assignment of HBx(101–136) were achieved by acquiring 2D ^1H–^{15}N HSQC, 3D HNCO, CBCA(CO)NH and HNCACB experiments. The Hα chemical shift resonances of HBx(101–136) were obtained from a 3D HNHA experiment [26]. Proton chemical shifts were referenced directly to DSS at 0 ppm, and ^{13}C and ^{15}N chemical shifts were referenced indirectly to the absolute frequency ratios $^{15}N/^1H = 0.101329118$ and $^{13}C/^1H = 0.251449530$, respectively [27]. All data were processed using TopSpin 3.1 and analyzed by Sparky ver. 3.115 [28]. The secondary structure was predicted by the program chemical shift index (CSI) [29] and the comparison result of the Hα chemical shift resonances of HBx(101–136) in 50% TFE with random coil reference values reported by Merutka et al. [30]. In the former method, the consensus (Hα, Cα, Cβ and CO) CSI values were used.

3. Results

3.1. Sample preparation and CD spectroscopy of HBx(101–136)

To prepare non-labeled, ^{15}N-labeled, and $^{13}C/^{15}$N-labeled samples, the ubiquitin fusion protein system was used [24,25]. The isolated HBx(101–136) was purified to >94%, as judged by 15–20% tricine–SDS–PAGE (Fig. 1B). Yields of HBx(101–136) were approximately 3 mg per litter culture for non-labeled sample and approximately 0.3 mg per litter culture for the ^{15}N-labeled or $^{13}C/^{15}$N-labeled sample, respectively. The yield of ^{15}N- or $^{13}C/^{15}$N-labeled sample was 10-fold lower, and thus higher yields of ^{15}N- or $^{13}C/^{15}$N-labeled sample is desirable for determining the

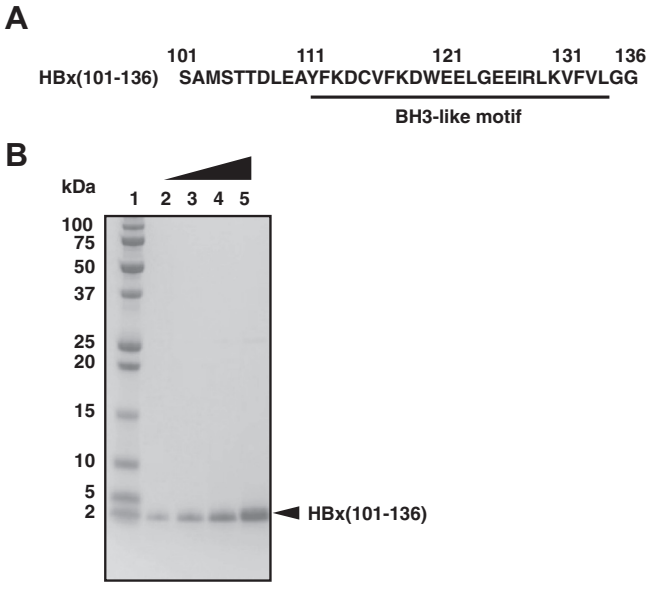

Fig. 1. (A) Amino acid sequence of HBx(101–136). The BH3-like motif is defined according to the Bak peptide used in the complex structure between Bak and Bcl-x_L [18]. (B) CBB stained 15–20% tricine–SDS–PAGE result of the purified HBx(101–136). Lane 1; Marker (Bio-Rad, CA, USA), lanes 2–5; 0.1, 0.3, 0.5 and 1 μg of HBx(101–136) were loaded into lanes 2–5.

three-dimensional structure of HBx(101–136). To further increase the levels of expression, a rich medium such as ^{15}N- or ^{13}C/^{15}N-labeled CHL medium (Chlorella Inc., Tokyo, Japan) should be potentially helpful, as previously reported by our group for the expression of the ^{15}N-labeled magainin 2 F5Y/F16W peptide where a yield of 1.2 mg per litter culture was obtained [25].

The secondary structure of HBx(101–136) was evaluated by CD spectroscopy (Fig. 2). The CD spectrum showed that HBx(101–136) is a random coil in aqueous solution (open circles, Fig. 2). We next examined the effect of TFE on the secondary structure of HBx(101–136). The CD spectra of HBx(101–136) in the presence of TFE exhibited well-defined double minima at 208 and 222 nm, which is characteristic of an α-helix conformation. Thus, it was revealed that HBx(101–136) forms an α-helix in the presence of TFE.

3.2. NMR spectroscopy and structural properties of HBx(101–136)

To determine the helical region of HBx(101–136) in the presence of 50% TFE, we performed the heteronuclear NMR experiments in 50% TFE at 25 °C. Fig. 3A shows the 2D ^{1}H–^{15}N HSQC spectrum of 0.3 mM ^{13}C/^{15}N-labeled HBx(101–136) in the presence of 50% TFE, which demonstrates good signal dispersion. In this spectrum, all of the backbone amide signals of HBx(101–136) could be observed, with the exception of the two N-terminal residues, Ser101 and Ala102. In addition, the side-chain indole signal of the tryptophan (W120) of HBx(101–136) was observed (Fig. 3A). The backbone ^{1}H, ^{13}C, and ^{15}N chemical shift resonances (NH, Hα, Cα, Cβ and CO) of HBx(101–136) were assigned using 3D HNCO, CBCA(CO)NH, HNCACB and HNHA experiments. Ninety-four percent of the backbone resonances could be assigned.

Reduced (free) and oxidized (forming disulfide bonds) cysteine residues can be distinguished by the Cβ chemical shifts [31]. The mean Cβ chemical shift value of free cysteine residues is 28.3 ± 2.2 ppm, whereas that with disulfide bonds is 40.7 ± 3.8 ppm [31]. The Cβ chemical shift value of Cys115 of HBx(101–136) is 27.1 ppm under the NMR conditions used (Fig. 3B), indicating that this cysteine is in the reduced state. Thus, HBx(101–136) does not form an intermolecular disulfide bond under the NMR conditions used.

The secondary structure of HBx(101–136) in 50% TFE was predicted from the results of the program CSI [29] by using the chemical shifts of Hα, Cα, Cβ and CO and the deviation of the Hα chemical shift values with respect to those in random coil reported by Merutka et al. [30]. No correction was performed for the effect of TFE, since changes in the Hα chemical shift values by TFE were generally negligible [30]. The former method showed that HBx(101–136) forms an α-helix structure from L108 to L134, whereas the latter method indicated that the α-helix region exists between residues L108–V133 (Fig. 4A). Both results are similar. Here, we found that HBx(101–136) adopts an α-helical structure under the NMR conditions, and the helical region is residues L108–L134, which includes the BH3-like motif consisting of residues Y111–L134.

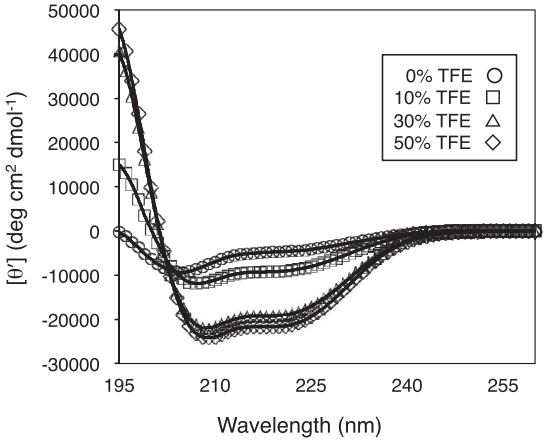

Fig. 2. CD spectra of HBx(101–136) at 0.03 mM in 20 mM potassium phosphate (pH 6.8) and 1 mM DTT at 25 °C, in the presence of 0% (open circle), 10% (open square), 30% (open triangle) and 50% (open rhombus) TFE.

4. Discussion

In this study, we revealed that HBx(101–136) containing the BH3-like motif is a random coil in aqueous solution, whereas residues L108–L134 adopt an α-helical structure in the presence of 50% TFE. This result is consistent with reports on other BH3 peptides. For example, the Harakiri BH3 peptide (residues 22–53) adopts an α-helix spanning residues 29–51 in TFE, whereas it is a random coil in aqueous solution [23]. In addition, it has been demonstrated that the formation of an α-helix by BH3 peptides is important for its interaction with Bcl-x_L [19]. Many structural studies of the BH3 peptide–Bcl-x_L complex have revealed that the BH3 peptide forms an amphipathic α-helix when it binds to the common binding site of Bcl-x_L [18–21]. Our preliminary NMR titration experiments using HBx(101–136) and Bcl-x_L have shown that HBx(101–136) binds to the common BH3-peptide binding site of Bcl-x_L (Kusunoki et al., unpublished data). Thus, our results strongly suggest that HBx interacts with Bcl-x_L via the BH3-like motif forming an α-helix structure in a similar manner to the interaction between the BH3 peptides and Bcl-x_L [18–21].

In the complex structures of the BH3 peptide and Bcl-x_L, the highly conserved leucine residue of the LXXXGDE sequence (X stands for any amino acid) in the BH3 motif makes critical contacts with residues in the hydrophobic groove of Bcl-x_L [18–21]. For example, a mutation of L78A in the Bak peptide results in reduced binding affinity to Bcl-x_L by a factor of 800 [18]. On the other hand, tryptophan instead of leucine exists in the HBx BH3-like motif (WXXXGEE). Importantly, this tryptophan residue (W120) is present on the same side as residues G124 and I127 (Fig. 4B). These two resides, G124 and I127, are likely involved in the interaction with Bcl-2 and Bcl-x_L, because the double mutation (G124L/I127A) of HBx impaired its ability to bind to Bcl-2, Bcl-x_L and the Bcl-2 homolog CED-9 [13,14]. Here, we termed the side including hydrophobic residues V116, F117, W120, L123, G124, and I127 (the three residues underlined are indicated by closed triangles in Fig. 4B), as the putative Bcl-2/Bcl-x_L binding side.

The tumor suppressor p53 is known to induce apoptosis by both transcription-dependent and transcription-independent mechanisms [32,33]. The latter mechanism is achieved by the interaction with anti-apoptotic proteins (Bcl-2 and Bcl-x_L), through the DNA-binding domain [33,34] and/or transactivation domain (TAD) of p53 [35,36]. Interestingly, the p53 TAD binds to Bcl-x_L in a similar manner to the interaction between the BH3 peptides and Bcl-x_L. The p53 TAD contains two conserved ΦXXΦΦ motifs (Φ indicates a bulky hydrophobic residue and X stands for any other residue), which consist of residues 15–29 (termed p53TAD1) and residues 39–57 (termed p53TAD2). For example, the p53TAD1/Bcl-x_L complex model, based on NMR experiments, showed that the hydrophobic side chains of F19, L22, W23 and L26 (the first three residues are in the ΦXXΦΦ motif) of p53TAD1 directed into the hydrophobic groove of Bcl-x_L [35]. Moreover, the p53TAD2/Bcl-x_L complex model demonstrated that the hydrophobic residues, I50, W53 and F54, in the ΦXXΦΦ motif are key determinants for binding to Bcl-x_L [36]. Both peptides contain tryptophan residues that are involved in the interaction with Bcl-x_L. Therefore, it is postulated that W120 of HBx is involved in the interaction with Bcl-x_L. Further structural

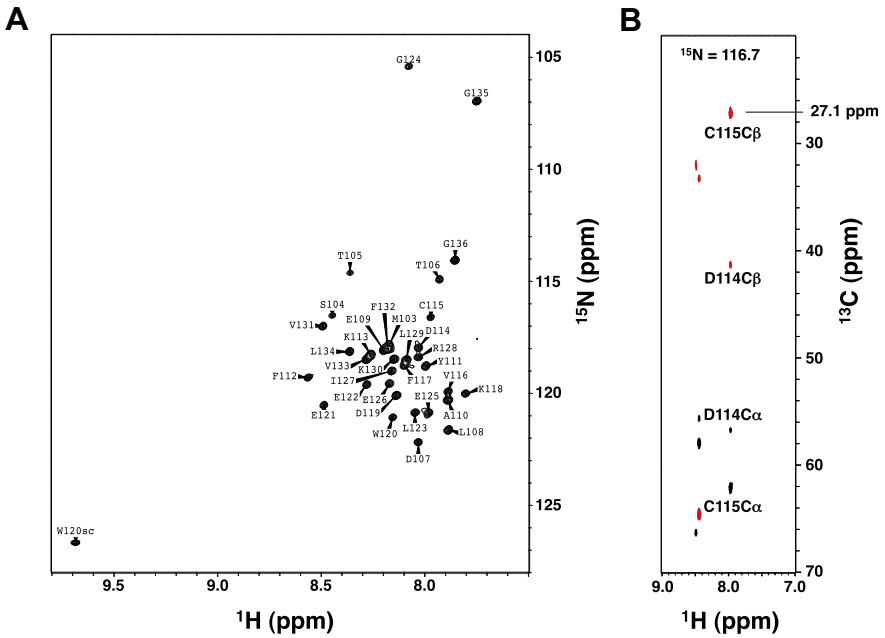

Fig. 3. NMR spectrum of HBx(101–136). (A) 2D ^{1}H–^{15}N HSQC spectrum of approximately 0.3 mM ^{13}C/^{15}N-labeled HBx(101–136) in the presence of 50% TFE. The assignment is labeled with each amino acid name and residue number. (B) A strip plot from the 3D HNCACB spectrum of C115 of HBx(101–136). The Cβ chemical shift of C115 is 27.1 ppm.

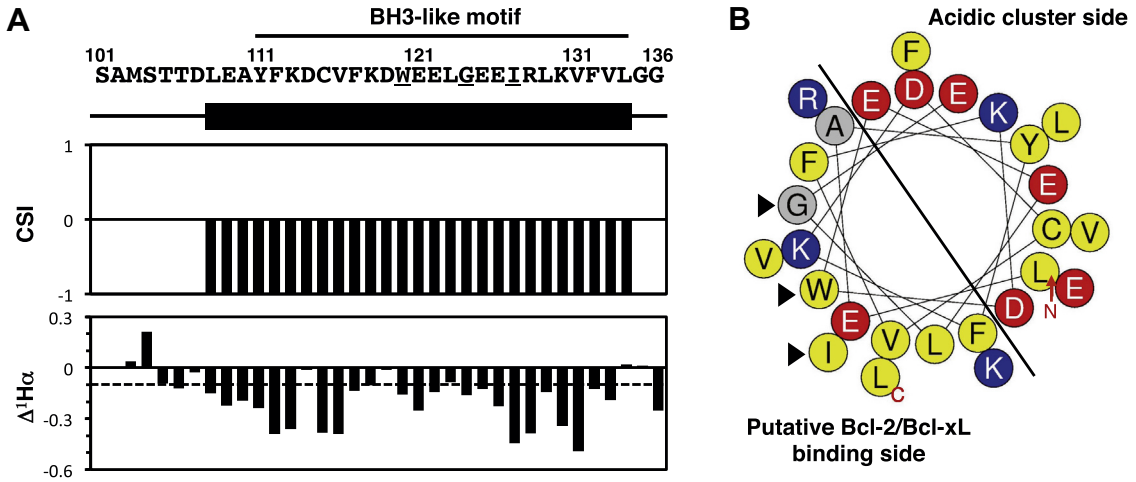

Fig. 4. Secondary structure diagram of HBx(101–136). (A) Secondary structure of HBx(101–136) in the presence of a 50% TFE solution was predicted from the program chemical shift index (CSI) [29] (top panel) and the comparison result of the Hα chemical shifts of HBx(101–136) in 50% TFE (bottom panel) with random coil reference values reported by Merutka et al. [30]. In top panel, the consensus CSI values are divided into the three-state (−1, 0, +1) index. The negative (−1), zero (0) and positive (+1) values indicate a tendency of the residues to adopt α-helix, random coil, and β-sheet secondary structures, respectively. In the bottom panel, the dashed line shows the standard threshold value of ΔHα (−0.1 ppm), and <−0.1 ppm indicates the tendency of α-helix. The closed rectangle under the amino acid sequence shows a helix region, L108–L134, of HBx(101–136) predicted by the above two methods. (B) Helical wheel diagram of the HBx BH3-like motif. Here, we termed the hydrophobic residue-rich side, as a putative Bcl-2/Bcl-x_L binding face, whereas the opposite side consists of an acidic cluster. The closed triangles show W120, G124 and I127, and G124 and I127 of HBx are likely involved in the interaction with Bcl-2 and Bcl-x_L [13,14]. The N and C indicate the N-terminal residue (L108) and the C-terminal residue (L134), respectively. The helical wheel diagram was obtained from the web server HELIQUEST [37].

and biochemical studies will help to confirm the role of this tryptophan residue in the interaction with Bcl-2 and Bcl-x_L.

The helical wheel diagram indicates that there is an acidic cluster on the opposite face of the putative Bcl-2/Bcl-x_L-binding side (Fig. 4B), suggesting that the acidic cluster may be involved in the interaction with other proteins containing basic amino acid rich surfaces. For example, HBx is known to interact with p53 [10–12] and the region of residues 102–136 of HBx reportedly interacts with both the DNA-binding domain and the oligomerization domain of p53 [12]. The interaction between HBx and p53 is also thought to contribute to the early stages of the development of HCC [10,11]. Therefore, this interaction mode is of great interest, and it is highly expected that the acidic cluster of the HBx BH3-like motif may be partly involved in the binding to the p53 DNA-binding domain that contains a basic amino acid rich surface.

In conclusion, we revealed that (i) the BH3-like motif of HBx can form an α-helix structure. (ii) Residues V116, F117, W120, L123, G124 and I127 are located on the one face of the α-helix, and this face represents a putative Bcl-2/Bcl-x_L binding side, whereas an acidic cluster is present on the opposite face. Our results imply that

the BH3-like motif of HBx may be involved in the interaction with Bcl-2 and Bcl-x_L through the α-helical structure and hydrophobic residues.

Acknowledgments

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