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Identification of the protein–protein contact site and interaction mode of human VDAC1 with Bcl-2 family proteins☆

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Abstract

Bcl-2 family of proteins plays differential roles in regulation of mitochondria-mediated apoptosis, by either promoting or inhibiting the release of apoptogenic molecules from mitochondria to cytosol. Bcl-2 family proteins modulate the mitochondrial permeability through interaction with adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC), ADP/ATP exchange, or oxidative phosphorylation during apoptosis. Although the mitochondrial homeostasis is affected by the relative ratio of pro- and anti-apoptotic Bcl-2 family members, the molecular mechanism underlying the release of mitochondrial intermembrane proteins remains elusive. Here we reported the biochemical evidence that both pro-apoptotic Bax and anti-apoptotic Bcl-X_L might simultaneously contact the putative loop regions of human VDAC1, and the existence of VDAC1–Bax–Bcl-X_L tertiary complex *in vitro* suggested that VDAC1 channel conformation and mitochondrial permeability could be determined by the delicate balance between Bax and Bcl-X_L.

VDAC, the most abundant protein of the outer mitochondrial membrane (OMM), forms an aqueous channel that is slightly anion-selective in the "open" state and cation-selective in the "closed" states [1]. Secondary and tertiary structure analyses predict that VDAC channel adopts a conserved β-barrel conformation formed by 16 β-strands reminiscent of bacterial porins [2–5]. The N-terminal globular α-helix and putative flexible loop regions (amino acids 104–120 and 150–169) point to the cytoplasmic direction [2,3,6]. VDAC channel plays a critical role in energy metabolism and mitochondrial homeostasis by permeating OMM to metabolites, including ADP and ATP, up to 1.5 kDa in size [1,7,8]. Moreover, association of VDAC with several kinases, such as hexokinase, creatine kinase, and glycerol kinase [9–12], suggests that VDAC might also possess a distinct role independent of its channel formation in control of metabolite trafficking across OMM. The regulated function of VDAC is further complicated by the findings that VDAC might be part of the multi-subunit complexes such as the mitochondrial permeability transition pore (mPTP) [8], translocase of the outer mitochondrial membrane (TOM) [13], or the contact site [14].

Recent studies strongly suggest that VDAC is involved in release of apoptogenic factors, such as cytochrome *c* [8,15–17], from mitochondria to cytosol to activate the apoptotic cascade, although the detailed molecular mechanisms of how VDAC forms a protein-conducting channel for the passage of cytochrome *c* are still in debate [18–20]. VDAC function could be further modulated by Bcl-2 family proteins [21–23], which

☆ *Abbreviations:* Bak, Bcl-2 homologous antagonist/killer; Bcl-X_L, Bcl-2-like 1 protein; GST, glutathione *S*-transferase; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VDAC, voltage dependent anion-selective channel.

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co-localize to OMM and may regulate mitochondrial membrane permeability and function of VDAC. Bcl-2 proteins can be divided into three different subfamilies based on Bcl-2 homology (BH) domains and function. The anti-apoptotic members (e.g., Bcl-2 and Bcl-X_L) typically possess BH1 through BH4 domains, while the pro-apoptotic members can be divided into two groups: those with BH1 through BH3 domains (e.g., Bax and Bak), and those with only BH3 domains (e.g., Bid, Bim, and Bad) [24]. Whether pro-apoptotic Bax functions in a VDAC-dependent [25–27] or VDAC-independent [28–31] fashion is still controversial, however, experiments with inhibitory antibodies against VDAC strongly indicate that the putative loop region of VDAC might contact Bax [27] and Bim [18] but not Bid [27] for the coordinated control of OMM permeability. On the other hand, experiments with isolated mitochondria suggest that Bcl-X_L directly affects the conformation and function of VDAC [20,32], although it is still unclear whether Bcl-X_L maintains VDAC in its open [20] or closed [32] conformation for metabolite exchange in receiving death stimuli. It is apparent, however, that BH4 domain is necessary and sufficient for Bcl-X_L to establish its inhibitory function in cytochrome *c* release and transmembrane potential ($\Delta\psi_m$) loss in both liposome and *in vivo* experiments [32].

Except for detailed genetic and biochemical studies on structure–function correlation of fungal VDACs [4,33–37], relatively little is known about structural determinants of human VDAC1 (hVDAC1) essential in apoptotic regulation. As a first step to address this issue, we mapped the interaction motifs of VDAC loop regions in protein–protein contact with Bax and Bcl-X_L. The results suggest for the first time that Bcl-2 family proteins, if not all, might utilize the common binding sites to form a tertiary complex with VDAC to execute their functions.

Materials and methods

Reagents. Shuttle plasmid pBDL-hVDAC1 carrying wild type human VDAC1 and its parental plasmid pBDL [26], anti-hVDAC1 antibodies Ab#25 and Ab#20 [27] were kindly provided by Dr. Tsujimoto (Osaka University, Japan). Yeast strain M22-2 lacking VDAC1 (*MAT* a *lys2 his4 trp1 ade2 por1*::LEU2 *ura3*) was a kind gift from Dr. M. Forte [38]. Anti-human VDAC monoclonal antibody (31HL) was obtained from Calbiochem. Glutathione (GSH)–Sepharose 4B beads were from Pharmacia. Talon metal affinity resins, anti-$His_{6\times}$ monoclonal antibody, SD minimal base, Trp DO supplement, and Protein A/G beads were from Clontech. Anti-Bcl-X_L (S-18) was purchased from Santa Cruz. Xanthine (X) and xanthine oxidase (XO) was obtained from Sigma. All other fine chemicals were purchased from Amresco.

Plasmids for overexpression of recombinant His-tagged hVDAC1 (rhVDAC1) and its mutants were mentioned elsewhere [39]. The paralleled deletional or truncational mutations lacking N-terminus, loop regions, or C-terminus were also introduced into pBDL-hVDAC1 by site-directed mutagenesis (QuikChange, Strategene) as depicted in Fig. 1. Among them, ΔL1 and ΔL2 were made by replacement of amino acids 104–120 and 150–169 with Ala–Ala–Gly–Ala–Ala pentameric peptide, respectively.

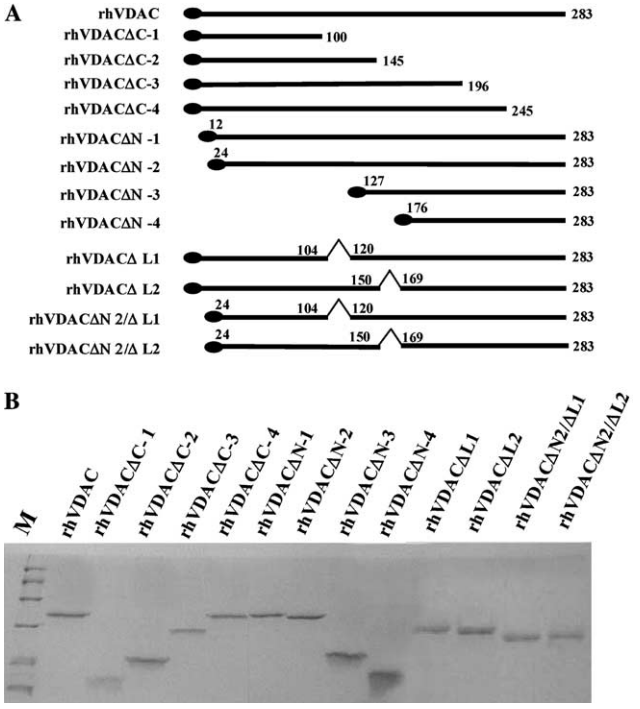

Fig. 1. Mutagenesis and purification of fully functional rhVDAC1 and its mutant variants. (A) Schematic representation of wild type rhVDAC1 and its deletion variants. rhVDAC1ΔN1–4 and rhVDAC1ΔC1–4 were N- and C-terminal truncation mutants, respectively. rhVDAC1-ΔL1 and -ΔL2 were mutants with corresponding loop region replaced by a pentameric peptide (Ala–Ala–Gly–Ala–Ala) to maintain the flexibility of rhVDAC1. Numbers are positions of starting and ending amino acids of the mutation. The hexameric histidine tag was shown as an oval. (B) Quality assessment of rhVDAC1 and mutants purified by affinity refolding method [39] in 15% SDS–PAGE and visualized by Coomassie blue staining. Equal amounts of various proteins were loaded.

Protein–protein interaction between hVDAC1 and Bcl-2 family proteins. Soluble recombinant wild type and mutant hVDAC1 proteins, fusion proteins of GST–Bcl-X_L, GST–Bcl-X_LΔTM (amino acids 210–233 of the transmembrane domain were deleted), and GST–BaxΔTM (amino acids 172–192 of the transmembrane domain were deleted) were prepared exactly as described [39].

GST pulldown assays were performed as described previously [26]. In brief, 150 nM rhVDAC1 and mutant variants were incubated with the same molar ratio of GST–Bcl-X_L, GST–BaxΔTM, or GST only proteins in 200 μl GST pulldown buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 1 mM EGTA, and 0.5% NP-40) for 1 h at 4 °C. Glutathione (GSH)–Sepharose 4B beads (20 μl) were added to the mixture and rocked gently for an additional 1 h at 4 °C. Beads were extensively washed with the same pulldown buffer before the proteins were eluted and separated in 12% SDS–PAGE. hVDAC1 was detected by Western blotting using anti-$His_{6\times}$ antibody (1:5000 in PBST/1% milk).

Antibody blocking experiments were performed in the same conditions as GST pulldown reactions except that 6 μg antibodies against N-terminus (31 HL), loop regions (Ab#20 and Ab#25) were incubated with hVDAC1 for 3 h at 4 °C before GST fusion proteins were added.

Peptide competition assays. HPLC purified BH3 (MGQVGRQLAI IGDDINRRY) and BH4 (SNRELVVDFLSYK LSQKGYS) peptides derived from human Bak or Bcl-X_L [40], and their loss-of-function mutant peptides BH3-A (MGQVGRQAAIIGDDINRRY, L78A) [41],

BH4-GG (SNREGGVDF LSYKLSQKGYS, L8G/V9G) [32] were obtained from SynPep (Dublin, CA). Increasing concentrations of various peptides as indicated were incubated with 50 nM rhVDAC1 in 250 μl GST pulldown buffer on ice for 1 h, and 60 nM Bcl-X_L was then added and incubated for another 1 h. About 3 μg anti-$His_{6\times}$ monoclonal antibody was added and the tubes were rocked gently for additional 1 h before 30 μl Protein A/G beads were applied to immunoprecipitated protein complexes. Co-immunoprecipitated Bcl-X_L was assessed by Western blotting using anti-Bcl-X_L antibody (S-18) and visualized by ECL SuperSignal system (Pierece).

Three-component His-tag pulldown assay was done similar to peptide competition assays except that the pulldown buffer was changed to PBS plus 0.5% NP-40, and 25 μl Talon beads instead of glutathione (GSH)–Sepharose 4B beads were used. Increasing amounts of recombinant Bax or Bcl-X_L as where indicated were used instead of peptides.

Cytochrome c release as the function of VDAC1. Various proteoliposomes were prepared as described previously [39] and 125 μl of each was incubated with or without 5 μM GST–Bcl-X_L in a total volume of 250 μl liposome buffer for 30 min at room temperature. X + XO was added to the final concentration of 0.1 mM and 20 mU/ml, respectively, according to the previous method [29]. FITC-cytochrome *c* released into the supernatant was quantified by the FITC fluorescence intensity (490 nm excitation and 510 nm emission, FluoroMax-2).

Yeast complementation assay. The −Trp SD and YPGA (2% glycerol as the carbon source) media were prepared according to the manufacturer's manual. Plasmids of pBDL-hVDAC1 and its mutant derivatives were transformed into M22-2 using LiAc method [42]. Transformants were selected on −Trp SD plates and streaked onto YPGA plate (2% glycerol as the carbon source). Viability at either 30 or 37 °C was scored 5 days post-inoculation.

Results and discussion

Dissection of functional domains of hVDAC1 by systematic mutagenesis

Human VDAC1 contains 283 amino acids, and genetic and biochemical experiments suggested that the inner wall of the channel could be formed by the amino acids comprising of 16 membrane-buried β-strands [2–5], which primarily determines the ion selectivity of the channel [43]. The N-terminal α-helix and the putative unstructured loop regions may wave around in cytoplasm. To determine the functional epitopes of hVDAC1 involved in permeability control, a series of truncation and deletion were introduced to yield mutant forms of hVDAC1 lacking N-terminus, loop regions or C-terminus (illustrated in Fig. 1A). By using the method described previously [39], soluble recombinant hVDAC1 (rhVDAC1) and the mutant variants were purified to near homogeneity as determined by SDS–PAGE method (Fig. 1B).

Recent liposomal and mitochondrial experiments indicated that reactive oxygen species (ROS), such as superoxide, may evoke cytochrome *c* release dependent on VDAC function but independent of either Bax or Bak translocation to mitochondria [29]. This result thus established a direct functional assay of VDAC-dependent permeabilization of OMM to cytochrome *c*. To assess whether the purified rhVDAC1 and its mutants are fully functional in gating cytochrome *c* release, VDAC proteoliposome was constituted to assess FITC-conjugated cytochrome *c* release in response to superoxide generated by xanthine plus xanthine oxidase reaction (X + XO; Fig. 2A). The proteoliposome encapsulated with FITC-cytochrome *c* was not leaky since it yielded a base-line variation of fluorescence in the presence or absence of ROS. Therefore, fluorescence intensities in the supernatant could therefore serve as the indicator of cytochrome *c* release. X + XO treatment led to more than 3-fold increase in fluorescence intensity for wild type rhVDAC1, in good agreement with the previous report using mitochondrial VDAC [29]. Mutant hVDAC1 of N-terminal truncation (ΔN1, ΔN2) and deletion of the loop regions (ΔL1, ΔL2) also yielded the similar fold of increase of cytochrome *c* release in the same condition. However, relatively drastic alteration within the barrel region of hVDAC1 (ΔN3–4 and ΔC1–4) abolished its pore forming capability as indicated by irresponsiveness to X + XO. Such defect in cytochrome *c*

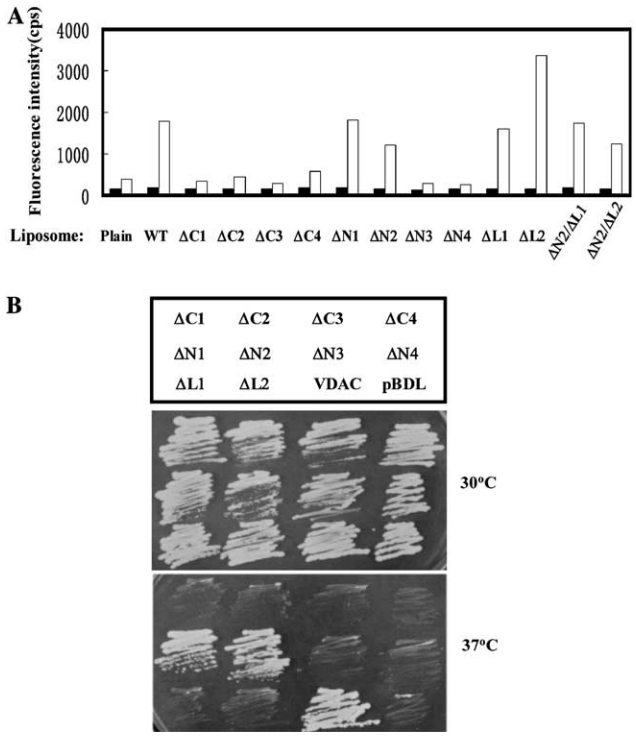

Fig. 2. Functional mapping of hVDAC1 domains. (A) FITC-cyto *c* released from various rhVDAC1 proteoliposomes as indicated in the absence (black bars) and presence (empty bars) of X + XO was quantified by the fluorescence intensity (490 nm excitation and 510 nm emission, FluoroMax-2). Data represented the average of three independent experiments. (B) Complementation of yeast VDAC1 deficiency by human VDAC1 and its mutant variants. Yeast cells M22-2 transformed with plasmid pBDL-hVDAC1 or various mutants as indicated in the grid were streaked to YPGA plates (glycerol as non-fermentable carbon source) and grown at either 30 °C (permissive temperature, top panel) or 37 °C (non-permissive temperature, bottom panel). Viability was scored 5 days post-streaking.

release of the corresponding VDAC mutants was not due to their failure to insert into lipid bilayers either, because Western blot against proteoliposomes showed a near equivalent encapsulation of wild type and mutant rhVDAC1 derivatives (data not shown). This result indicated that renatured rhVDAC1 sustains biological function in response to ROS as compared to that of VDAC in mitochondria. This result further suggested that the N-terminal and loop regions of VDAC1 would be dispensable for permeability control of cytochrome *c*, while the rest of the regions of VDAC might be essential in maintaining the structural integrity, reminiscent of previous mutational studies on ion selectivity of yeast VDAC1 [43].

Previous studies also showed that yeast VDAC1-depleted mitochondria displayed a highly reduced permeability of the outer membrane, and hVDAC1 could functionally complement the slow growth phenotype at non-permissive temperature [31,38,44]. This therefore provides a convenient assessment of hVDAC1 permeability in a surrogate genetic system. To characterize whether various hVDAC1 mutants could functionally complement yeast VDAC1 deficiency, they were transformed into the yeast strain M22-2 [38] lacking endogenous VDAC1. All the mutant hVDAC1 transformants were able to grow on a non-fermentable carbon source (glycerol) at permissive temperature (Fig. 2B, top panel). On the other hand, ectopically expressed wild type hVDAC1, hVDAC1-ΔN1, and -ΔN2 could fully rescue the growth at 37 °C, the rest of mutants failed to do so (Fig. 2B, bottom panel). It was not surprising that hVDAC1ΔN3–4 and VDAC1ΔC1–4 could not support the growth at 37 °C because the truncated regions could be essential to maintain the structural integrity thus ion-selectivity of the channel [43,45]. However, it is rather surprising that hVDAC1ΔL1 and hVDAC1ΔL2 failed to complement the growth deficiency at 37 °C, as these mutants were most likely disposable for gating cytochrome *c* release in the proteoliposome experiments. A likely explanation would be that these cytosol exposed loops might provide the binding sites for various kinases involved in metabolite exchange [9–12], deletion of these regions therefore would inevitably cause lethal respiration defect. The N-terminus was shown to be dispensable for hVDAC1 in metabolite homeostasis because hVDAC1DN1 and hVDAC1DN2 grew as well as the wild type hVDAC1. This notion was supported by previous liposomal assays studies in which the amphipathic α-helical N-terminal end was not involved in channel gating [46] although it might be rather mobile [5].

Functional mapping of protein–protein interaction epitopes of hVDAC1 with Bax and Bcl-X_L

Mutational analysis suggested that the regions interspersed the N-terminus and the putative loops of hVDAC1 were involved in maintenance of the β-barrel integrity, therefore these membrane-buried regions would likely be inaccessible to modulation of Bcl-2 family proteins. However, the putative loop regions of hVDAC1, required for metabolite exchange, might also serve as a potential contact site for Bax and Bcl-X_L. Indeed, Bcl-X_L could maintain VDAC in open configuration to mediate metabolite exchange essential for coupled respiration and survival during serum withdraw [20,47,48]. To assess the contact sites of hVDAC1 in making protein–protein interaction with Bcl-2 family members, wild type hVDAC1, the N-terminal truncation (hVDAC1-ΔN1 and -ΔN2), and loop deletion (hVDAC1-ΔL1 and -ΔL2) mutants were tested for their ability to interact with Bax (panel (i)) or Bcl-X_L (panel (ii)) in GST pulldown experiments (Fig. 3A). While hVDACΔN1 yielded the similar efficiency in interaction with Bax or Bcl-X_L as wild type hVDAC1, hVDACΔN2, hVDACΔL1, and hVDACΔL2 showed much decreased affinity in binding to Bax or Bcl-X_L (panels (i) and (ii)), with hVDACΔN2 and hVDACΔL1 being more severe. This result therefore confirmed the previous speculation that Bax might utilize two loops in mediating cytochrome *c* release [27]. Our results suggested for the first time that this same region might

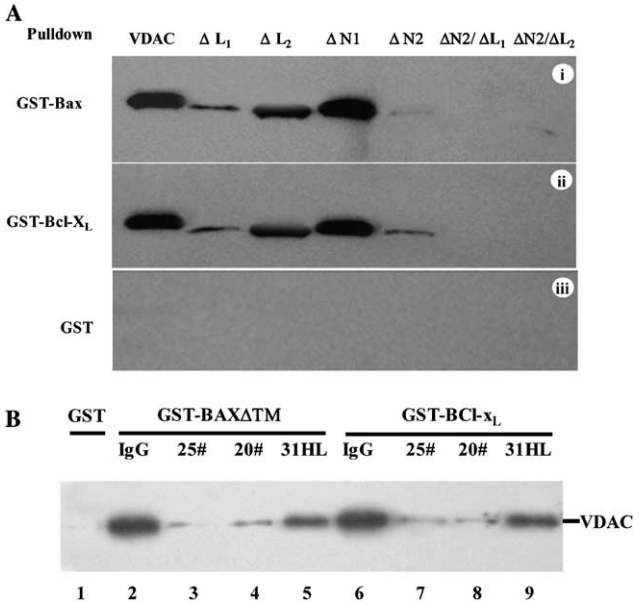

Fig. 3. Bax and Bcl-X_L interact with hVDAC loop regions. (A) Interaction between hVDAC1 and Bax and Bcl-X_L. About 150 nM rhVDAC1 or mutant proteins were first incubated with the same molar ratio of GST–BaxΔTM (panel (i)), GST-Bcl-X_L (panel (ii)) or GST alone (panel (iii)) in 200 μl of GST pulldown buffer at 4 °C for 1 h, 20 μl glutathione–Sepharose 4B beads were then added to pulldown associated complex, and rhVDAC1 fractions were assessed by Western blotting using anti-$His_{6\times}$ antibody. (B) Antibody blocking assay. The reactions were performed in the same condition as in (A) except that 6 μg antibodies (Ab#20, Ab#25, or porin 31HL) were first incubated with wild type rhVDAC1 at 4 °C for 3 h before GST fusion proteins of Bax or Bcl-X_L were added.

participate in protein–protein contact with Bcl-X_L as well. The N-terminal (amino acids 12–24) of VDAC1 could potentially modulate the conformation thus accessibility of VDAC1 to Bax or Bcl-X_L because double mutants (hVDAC1ΔN2/ΔL1 and hVDAC1ΔN2/ΔL2) showed more pronounced defect in binding to Bax or Bcl-X_L (Fig. 3A).

The reduced binding of mutant hVDAC1 to Bax or Bcl-X_L as observed above could be caused by unfavorable conformational alteration introduced by the deletion of the loop or the N-terminus of hVDAC1. To rule out this possibility, antibody blocking assay was performed in which full length rhVDAC1 was incubated with antibodies specifically against the N-terminus (31HL) or the two loops (Ab#25 against the first loop, or Ab#20 against the second loop) before GST–Bcl-X_L or GST–Bax fusion proteins were added (Fig. 3B). While the non-specific IgG could not interfere with the binding of hVDAC1 to Bax or Bcl-X_L (lanes 2 and 6), antibodies against the loops effectively block the interaction between hVDAC1 and Bcl-X_L or BaxΔTM (lanes 3–4 and 7–8). Occupation of the N-terminal hVDAC1 by monoclonal antibody 31HL yielded a moderate decrease in association of these two molecules (lanes 5 and 9). Therefore, our results strongly indicated for the first time that hVDAC1 might utilize the same loop regions in making protein–protein interaction with Bax and Bcl-X_L.

The interaction between hVDAC1 and Bcl-2 family members was further investigated in VDAC proteoliposome assays. Bax or Bcl-X_L proteoliposomes did not respond to X + XO (Fig. 4A), which suggested that the potential Bax or Bcl-X_L pore, if there existed, could not be opened by ROS in our particular assay conditions. For hVDAC1 proteoliposome, addition of Bcl-X_L without transmembrane domain (Bcl-X_LΔTM) was less effective in closing the VDAC1 channel compared to the full length Bcl-X_L, suggesting a requirement of membrane insertion for Bcl-X_L to function. Surprisingly, addition of Bax to rhVDAC1 proteoliposome yielded no observed increase in cytochrome c release, which might reflect a subtle requirement for Bax of particular mitochondrial cardiolipin contents not present in the soybean phospholipid (Soybean type-IV) used in our experiments [49]. Considering the observation that a unique chimeric channel by VDAC1 and Bax yielded no ion selectivity or voltage-dependent modulation in electrophysiological analysis [50], only interaction between Bcl-X_L and VDAC1 was further examined (Fig. 4B). In the presence of X + XO, addition of Bcl-X_L normally yielded >3-fold inhibition of cytochrome *c* release for wild type hVDAC1 liposome (Figs. 1C and 4B). Such antagonistic effect by Bcl-$X_L$against the VDAC channel was decreased by more than 2-fold with the mutant hVDAC1ΔL1, and 1.5-fold with hVDAC1ΔL2 liposomes (Fig. 4B), suggesting that these

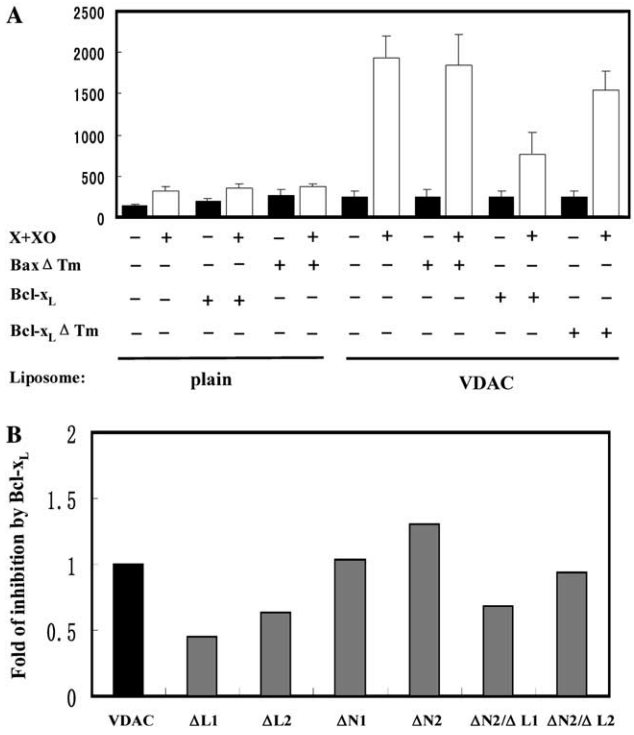

Fig. 4. The loop regions of hVDAC1 mediated permeability control of Bcl-X_L to cytochrome *c*. (A) FITC-cytochrome *c* encapsulated plain liposome or wild type rhVDAC1-incorporated proteoliposome was incubated with 5 μM Bcl-X_L, Bcl-X_LΔTM, BaxΔTM, or suspension buffer as a blank at 25 °C for 30 min. FITC-cyto *c* released into the supernatant in the absence (black bars) or presence (empty bars) of X + XO was measured in a FluoroMax-2 fluorometer ($\lambda_{excitation}$ = 490 nm, $\lambda_{emission}$ = 510 nm). Data were the average of four independent experiments. (B) Proteoliposomes of rhVDAC1 and various mutants as indicated were incubated with or without 5 μM Bcl-X_L in the presence of X + XO. The *Y*-axis represented the fold of inhibition of FITC-cyto *c* release by Bcl-X_L, which was the ratio of fluorescence with Bcl-X_L and without Bcl-X_L. Fluorescence of rhVDAC1 mutants was normalized to that of wild type rhVDAC1. Data represented the average of two independent experiments.

regions might be required for Bcl-X_L to function. Alteration at the N-terminal hVDAC1 (hVDAC1ΔN1) gave rise to no apparent defect in response to Bcl-X_L, or even enhancement of cytochrome *c* release for hVDAC1ΔN2, suggesting a possible repressive function of the hVDAC1 N-terminal domain on Bcl-X_L accessibility. This notion was buttressed by the observation that simultaneous deletion of N-terminal and loop region (hVDAC1ΔN2/ΔL1 and hVDAC1ΔN2/ΔL2) partially restored the inhibitory effect of Bcl-X_L (Fig. 4B).

Mode of interaction between VDAC and Bcl-2 family proteins

It has long been acknowledged that the outcome of mitochondria-mediated apoptotic control is fine-tuned by the balance between Bax and Bcl-X_L [51]. Whether direct physical contact with VDAC is required [27,44] or not [31] for Bax/Bcl-X_L to function in surrogate yeast

systems remains controversial. It is intriguing, based on our results, that both Bax and Bcl-X_L might contact the same loop regions within hVDAC1 in both GST pull-down and antibody blocking experiments. Considering the fact that BH3 domain is not required for Bax to interact with VDAC1 [52], it is reasonable to propose that simultaneous binding of Bax and Bcl-X_L to hVDAC1 loops could be bridged by interlocking of BH3 domain of Bax to the hydrophobic cleft of Bcl-X_L [40]. To test whether a VDAC1–Bax–Bcl-X_L tertiary complex exists, three-component co-immunoprecipitation experiments were performed in which Bax or Bcl-X_L was first incubated with equal molar ratio of rhVDAC1 followed by titration into increasing concentrations of Bcl-X_L or Bax, respectively. To our expectation, affinity pulldown by Talon beads against the His-tag fused to rhVDAC1 (Fig. 1A) could effectively pull down Bax and Bcl-X_L concomitantly (Fig. 5A, lanes with concentrations of Bax or Bcl-X_L greater than 10 nM). To our surprise, however, sub-stoichiometric amount of Bcl-X_L (panel (i)) or Bax (panel (ii)) (less than 40 nM for both proteins) first enhanced the binding of the opponent protein to rhVDAC1, as the concentration reached 40 nM, it started to compete against the opponent for binding to rhVDAC1. Excessive amount of Bax or Bcl-X_L (>80 nM) dramatically vied against each other in association with rhVDAC1 (Fig. 5A, panels (i) and (ii)). This result therefore suggested that pre-occupation of VDAC1 loop regions by either Bax or Bcl-X_L did not interfere with the other molecule to join in, and heterodimeric interaction between Bax and Bcl-X_L could stabilize the tertiary complex. This result further suggested that the conformation of the resulting VDAC1–Bax–Bcl-X_L complex would depend on the ratio between Bax and Bcl-X_L, because excessive amount of one of the factors would eventually sequester the opponent therefore determine hVDAC1 confirmation.

To further dissect the binding kinetics, peptide interference experiments were performed to assess the association of Bcl-X_L with hVDAC1 (Fig. 5B). Increasing amounts of BH3 peptide of Bak or BH4 peptide of Bcl-X_L enhanced the binding of Bcl-X_L to hVDAC1 (panels (i) and (iii)). Moreover, this effect was specific since either mutant peptide BH3-A or BH4-GG failed to do so (panels (ii) and (iv)). The increased binding of Bcl-X_L with hVDAC1 could be due to altered conformation of Bcl-X_L induced by BH3/Bcl-X_L interaction that would favor the association with VDAC1, despite crystallographic studies showed that overall conformation of Bak BH3/Bcl-X_L complex was similar to that of uncomplexed Bcl-X_L [40]. This notion is supported by recent finding that BH3 peptide derived from Bax exerts its pro-apoptotic activity by displacement of endogenous Bax from heteromeric anti-apoptotic Bcl-X_L [53,54], and it requires the correct insertion of Bax to OMM. It is highly possible that, while BH3/Bcl-X_L occupies one of the loops of VDAC1, the displaced Bax could still associate with another loop of VDAC1. Such BH3–Bcl-X_L–Bax–VDAC quadruplex, possibly adopting a different configuration than VDAC1–Bcl-X_L binary complex, and could be an active spatial arrangement for pro-apoptotic action by Bax. On the other hand, addition of BH4 peptide of Bcl-X_L could induce VDAC1 conformational change, which would benefit the tighter association of Bcl-X_L with VDAC. Indeed, it has been shown that BH4 domain of Bcl-X_L itself is sufficient to block VDAC-dependent cytochrome *c* release [32], and such action by BH4 peptide did not rule out the possible collaborative interaction between BH4 peptide with endogenous Bcl-X_L. Based on these observations, it is intriguing to propose that the balance between Bax and Bcl-X_L might influence the confor-

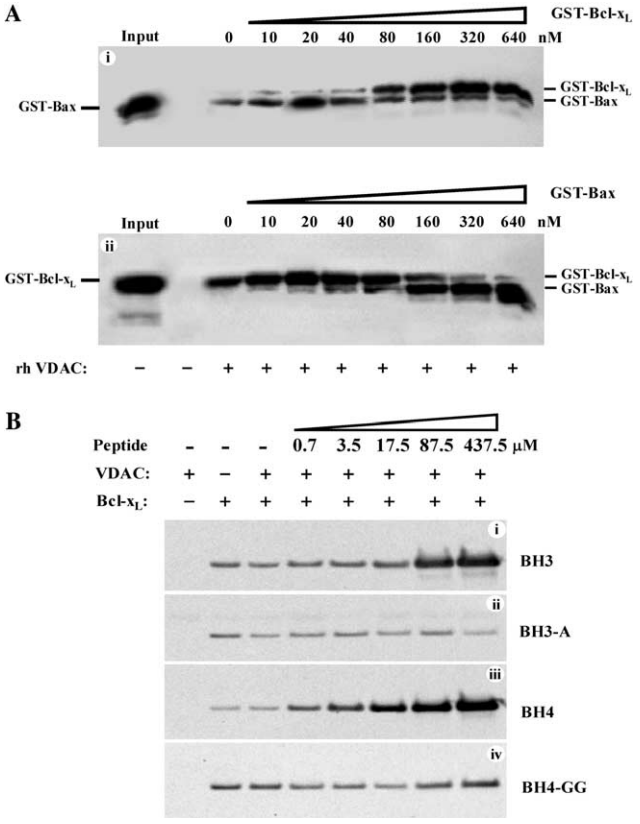

Fig. 5. Interaction mode of Bax and Bcl-X_L in contact with hVDAC1. (A) Eighty nanomolars of GST–BaxΔTM was first incubated with equal amount of rhVDAC1, increasing concentrations of GST–BaxΔTM were then added, and protein complex was then co-immunoprecipitated with anti-$His_{6\times}$ antibody and Western blotted with anti-GST antibody (panel (i)). Reciprocal of three-component co-immunoprecipitation was done similarly but using 80 nM GST–Bcl-X_L to react with rhVDAC1 first and titrated into increasing amounts of GST–Bcl-X_L (panel (ii)). (B) Fifty nanomolars of rhVDAC1 was mixed with increasing concentrations of BH3 (panel (i)), BH3-A (panel (ii)), BH4 (panel (iii)), and BH4-GG (panel (iv)) peptides as indicated in 250 μl GST pulldown buffer, and 60 μM Bcl-X_L was then added to incubate for an additional 1 h before anti-$His_{6\times}$ antibody was used to pulldown VDAC1–Bcl-X_L complex. Western blot using anti-Bcl-X_L antibody was done to detect the presence of Bcl-X_L.

mation thus activity of VDAC1, and no matter which of these two factors prevails, the VDAC1–Bax–Bcl-X_L tertiary complex might reflect a transitional state required for formation of VDAC1–Bax or VDAC1–Bcl-X_L complex. This hypothesis is now under evaluation.

Whether Bcl-2 family proteins execute their functions through mPTP is still under debate, largely due to different model systems and different approaches that various laboratories applied. Our data strongly suggested, at least in *in vitro* reactions, that Bcl-X_L and Bax could make direct and specific protein–protein contact with hVDAC1 loop regions, and the N-terminal hVDAC1 could modulate this interaction to certain extent. Mutations at the loop regions yielded lethality in yeast complementation experiments, which suggest that this region of VDAC1 could be involved in pleiotropic functions not only in interaction with Bcl-2 family members but also components of energy metabolic pathways. This phenotype limited us to otherwise thoroughly analyze the functionality of VDAC1/Bax/Bcl-X_L interaction in yeast. However, we provided convincing evidence for the first time that a functional VDAC1/Bax/Bcl-X_L heterotrimeric complex might exist, and VDAC1 conformation thus function could be fine-tuned by the ratio of Bax to Bcl-X_L. We noticed a potential two-step kinetic reaction between Bax and Bcl-X_L domains with VDAC1, i.e., low concentrations of Bax enhanced the association of Bcl-X_L with VDAC1, and the high concentrations of Bax promote the dissociation of Bcl-X_L from VDAC1, and vice versa. This could be caused by two conformation states of VDAC1 in binding to Bax and Bcl-X_L.

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