Communication

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Anja C. Gemperli, Stacey E. Rutledge, Abby Maranda, and Alanna Schepartz

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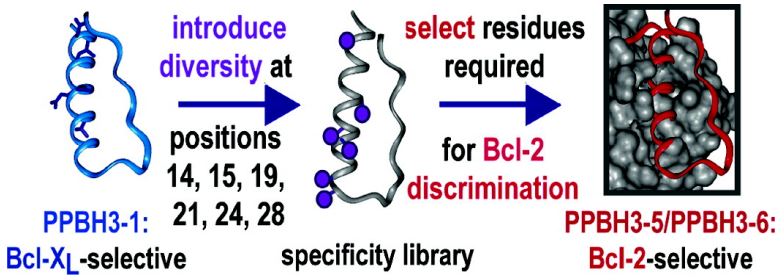

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Paralog-Selective Ligands for Bcl-2 Proteins

Anja C. Gemperli,[†] Stacey E. Rutledge,[†] Abby Maranda,[†] and Alanna Schepartz*[,†,§]

Departments of Chemistry and Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520

Received September 27, 2004; E-mail: alanna.schepartz@yale.edu

There is considerable interest in molecules that bind intra- or extracellular protein surfaces and inhibit protein−protein interactions.[1] Molecules with these properties have potential as validation tools or therapeutic leads for the vast array of proteins encoded by the human genome and can probe the functional relevance of molecular circuits that control the inner workings of the cell. Accurate interpretation of such chemical biology experiments, however, demands an exceedingly high and often elusive level of specificity, as the phenotypic readout will reflect the weighted integral of all cellular binding events, whether they involve the desired target or not. This caveat is especially important when closely related protein family members or paralogs are coexpressed and nonredundant.[2]

Previously we reported a miniature protein design strategy in which the well-folded structure of the pancreatic-fold polypeptide aPP presents α-helical or PPII-helical recognition epitopes found on protein−protein interaction surfaces.[4−6] The miniature proteins designed in this way can recognize even shallow protein clefts with high affinity and specificity and inhibit protein−protein interactions.[4f] One such miniature protein, PPBH3-1, binds the anti-apoptotic protein paralogs Bcl-2 and Bcl-X_L with nanomolar affinity and a $\Delta\Delta G = 1.2$ kcal·mol^{-1} preference for Bcl-X_L in vitro. Moreover, PPBH3-1 competes effectively with a peptide comprising the BH3 domain of the pro-apoptotic Bcl-2 protein Bak.[4d] Here we describe the evolution of PPBH3-1 into two new miniature proteins, PPBH3-5 and PPBH3-6, whose paralog specificity is reversed relative to PPBH3-1 (Figure 1A). PPBH3-5 and PPBH3-6 bind Bcl-2 with nanomolar affinity and a $\Delta\Delta G = 0.9-1.3$ kcal·mol^{-1} preference for Bcl-2 over Bcl-X_L. PPBH3-5 and PPBH3-6 may have unique applications as early examples of nonnatural ligands that interact selectively with Bcl-2 proteins.[7,8]

We began with a phage library of 5×10^8 PPBH3-1 variants whose sequences varied at six positions chosen to exploit subtle structural and electronic differences between the BH3-binding grooves of Bcl-X_L and Bcl-2 (Figure 1B).[9,10] These grooves are lined with remarkably similar side chains, with only three notable differences: Glu_{136} in Bcl-X_L is replaced by Arg_{129} in Bcl-2, Ala_{104} in Bcl-X_L is replaced by Asp_{111} in Bcl-2, and Leu_{108} in Bcl-X_L is replaced by Met_{115} in Bcl-2.[11] The limited sequence changes within the BH3-binding grooves belie significant functional differences between the two proteins: Bcl-2 and Bcl-X_L knock-out mice show dissimilar defects,[12] the proteins exhibit overlapping but distinct subcellular distributions,[13] and otherwise isogenic cell lines that overexpress Bcl-2 or Bcl-X_L respond differently to chemotherapeutic agents.[14]

We devised a positive/negative selection protocol to enrich the library with members that prefer Bcl-2 to Bcl-X_L. This protocol selected members that bound GST-Bcl-2_{1-205} (Bcl-2) with high affinity, eliminating those that also bound well to GST-Bcl-X_{L1-211} (Bcl-X_L). After seven rounds, the retention of the phage pool on Bcl-2-coated plates increased by 2000-fold relative to the initial pool. Two sequences that predominated among the 80 clones sequenced—PPBH3-5 and PPBH3-6—were synthesized and labeled with iodoacetamidofluorescein to generate PPBH3-5^{Flu} and PPBH3-6^{Flu}.

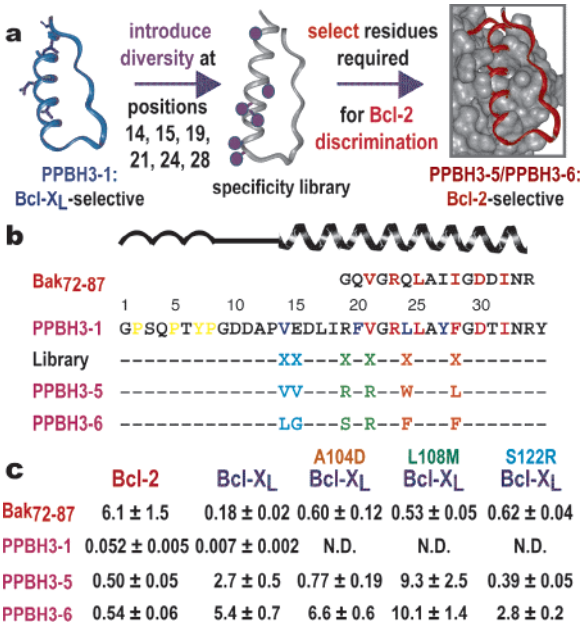

Figure 1. (A) Bcl-2-selective miniature protein evolution. (B) Alignment of Bak_{72-87}, PPBH3-1, the specificity library, PPBH3-5, and PPBH3-6. Residues in red contribute to the binding of Bcl-X_L by Bak_{72-87}; residues in blue and yellow contribute to aPP folding.[3] Positions varied in the library are indicated by X. (C) Equilibrium dissociation constants (μM) of complexes with Bcl-2 proteins.[4a] N.D. indicates not determined.

We used a direct fluorescence polarization assay to measure the affinity of PPBH3-5^{Flu} and PPBH3-6^{Flu} for Bcl-2 and Bcl-X_L. Each molecule bound Bcl-2 well, with equilibrium dissociation constants (K_d) of 505 and 543 nM, respectively (Figure 1C). These dissociation constants are 10-fold lower than that of the Bak_{72-87}·Bcl-2 complex ($K_d = 6.1 \pm 1.5$ μM) and 10-fold higher than that of the PPBH3-1^{Flu}·Bcl-2 complex ($K_d = 52 \pm 5$ nM).[4d] However, unlike Bak_{72-87} or PPBH3-1, both PPBH3-5 and PPBH3-6 prefer Bcl-2 to Bcl-X_L (Figure 2A,B). The equilibrium dissociation constants of the Bcl-X_L complexes of PPBH3-5 and PPBH3-6 were 2.7 ± 0.5 and 5.4 ± 0.7 μM, respectively, corresponding to paralog specificities of $\Delta\Delta G = -0.9 \pm 0.1$ and -1.3 ± 0.2 kcal·mol^{-1}. By contrast, both PPBH3-1 and Bak_{72-87}, as well as the lower affinity ligands PPBH3-2 and PPBH3-3,[4g] prefer Bcl-X_L with specificities of $\Delta\Delta G = +1.1 \pm 0.2$ and $+1.4 \pm 0.1$ kcal·mol^{-1}, respectively.[4d,g] Neither PPBH3-5^{Flu} nor PPBH3-6^{Flu} bound well to glutathione-S-transferase or bovine serum albumin ($K_d > 30$ μM) or to calmodulin ($K_d > 1$ mM). These results suggest that our

[†] Department of Chemistry.
[§] Department of Molecular, Cellular & Developmental Biology.

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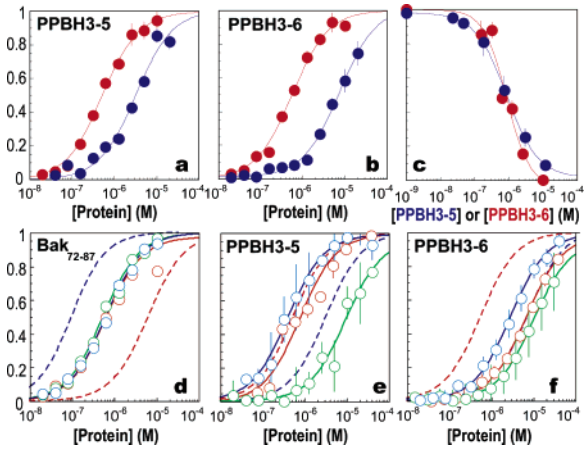

Figure 2. (a, b) Plot of the fraction of 25 nM PPBH3-5^{Flu} or PPBH3-6^{Flu} bound as a function of the concentration of Bcl-2 (red) or Bcl-X_L (blue) at 4 °C in PBS buffer (pH 7.4). (c) Plot of the fraction of 25 nM Bak$_{72-87}^{Flu}$ bound to Bcl-2 (8.5 μM) as a function of the concentration of PPBH3-5 or PPBH3-6. (d−f) Plot of the fraction of 25 nM Bak$_{72-87}^{Flu}$, PPBH3-5^{Flu}, or PPBH3-6^{Flu} bound as a function of the concentration of Bcl-X_L^{A104D} (red circles), Bcl-X_L^{L108M} (green circles), Bcl-X_L^{S122R} (blue circles), Bcl-X_L (blue dashed line), or Bcl-2 (red dashed line). Data were fit as described.[4d]

evolution protocol altered paralog specificity by >2 kcal·mol^{-1} through negative selection,[15] eliminating variants that bound well to Bcl-X_L as well as Bcl-2.

Two experiments were performed to investigate the binding modes of PPBH3-5 and PPBH3-6. First we performed competition fluorescence polarization experiments to assess whether PPBH3-5 and PPBH3-6 competed with fluorescently tagged Bak$_{72-87}^{Flu}$ for binding to Bcl-2 (Figure 2C). Both PPBH3-5 and PPBH3-6 competed well (IC_{50} = 742 ± 68 and 861 ± 279 nM, respectively).[17] These results indicate that Bcl-2 cannot interact simultaneously with Bak$_{72-87}^{Flu}$ and either PPBH3-5 or PPBH3-6 supports a model in which PPBH3-5 and PPBH3-6 bind Bcl-2 in or near the BH3-binding cleft.

To further investigate the binding modes of PPBH3-5 and PPBH3-6, we constructed a set of three Bcl-X_L variants (GST-Bcl-X_L^{A104D} (Bcl-X_L^{A104D}), GST-Bcl-X_L^{L108M} (Bcl-X_L^{L108M}), and GST-Bcl-X_L^{S122R} (Bcl-X_L^{S122R})), in which a single residue in the BH3-binding cleft was substituted with the corresponding residue from the BH3-binding cleft of Bcl-2. The three residues chosen represent the most notable differences among the residues lining the two BH3-binding pockets. As expected, Bak$_{72-87}^{Flu}$ bound each variant more poorly than it bound wild-type Bcl-X_L, with equilibrium dissociation constants between 0.53 ± 0.05 and 0.62 ± 0.04 μM (Figure 2D). The similarity of these values, and their intermediacy relative to those measured for the Bak$_{72-87}$·Bcl-2 and Bak$_{72-87}$·Bcl-X_L complexes, suggests that Bcl-X_L residues 104, 108, and 122 contribute equally to the observed preference of Bak$_{72-87}$ for Bcl-X_L.

By contrast, PPBH3-5^{Flu} bound two of these variants significantly better than it bound wild-type Bcl-X_L, and the free energy changes were magnified relative to those for Bak$_{72-87}$ (Figure 2E). The equilibrium dissociation constants of the PPBH3-5^{Flu}·Bcl-X_L^{A104D} and PPBH3-5^{Flu}·Bcl-X_L^{S122R} complexes were 0.77 ± 0.19 and 0.39 ± 0.05 μM, respectively, values virtually identical to those observed with wild-type Bcl-2, whereas the equilibrium dissociation constant of the PPBH3-5^{Flu}·Bcl-X_L^{L108M} complex was 9.3 ± 2.5 μM, virtually identical to that observed for wild-type Bcl-X_L. Interestingly, PPBH3-6^{Flu} did not bind any variant significantly better than it bound Bcl-X_L (Figure 2F). These data, taken with the competition experiments, suggest that PPBH3-5 interacts with Bcl-2 in a manner that mimics Bak$_{72-87}$, whereas PPBH3-6 interacts in an overlapping site or, perhaps, affects the binding of Bak$_{72-87}$ through allostery. Apparently, PPBH3-5 achieves paralog specificity by exploiting structural or electrostatic differences in the BH3-binding grooves of Bcl-2 and Bcl-X_L, whereas PPBH3-6 does so by exploiting alternative protein surfaces.

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Supporting Information Available: Procedures for phage display and fluorescence polarization assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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