Engineering a Protein Scaffold from a PHD Finger

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Summary

The design of proteins with tailored functions remains a relatively elusive goal. Small size, a well-defined structure, and the ability to maintain structural integrity despite multiple mutations are all desirable properties for such designer proteins. Many zinc binding domains fit this description. We determined the structure of a PHD finger from the transcriptional cofactor Mi2β and investigated the suitability of this domain as a scaffold for presenting selected binding functions. The two flexible loops in the structure were mutated extensively by either substitution or expansion, without affecting the overall fold of the domain. A binding site for the corepressor CtBP2 was also grafted onto the domain, creating a new PHD domain that can specifically bind CtBP2 both in vitro and in the context of a eukaryotic cell nucleus. These results represent a step toward designing new regulatory proteins for modulating aberrant gene expression in vivo.

Introduction

The design of new proteins with tailored binding properties has become an area of great interest in recent years. Designer proteins have considerable potential as both therapeutics and reagents for probing cellular function. Whereas some have approached this goal from the bottom up, designing new protein folds either rationally (Woolfson, 2001) or combinatorially (Streekt and Mayo, 1999), an alternative approach involves taking a preexisting protein fold and mutating a subset of residues to introduce a new function. Currently, monoclonal antibodies (mAbs) comprise the best-established example of this protein redesign strategy, and there are currently over 70 mAbs undergoing clinical trials (Glennie and Johnson, 2000).

We have set out to determine whether zinc binding domains may serve as suitable scaffolds onto which nonnative functions may be grafted. For example, we recently demonstrated that around 70% of the amino acids in a small zinc binding motif could be mutated to alanine without disruption of the fold (Sharpe et al., 2002). This “stripped-down” structure may be a suitable template onto which new functions could be grafted.

Another of our initial targets for this design process was the PHD (plant homeodomain) motif. PHD or leuke-mia associated protein (LAP) domains are found in more than 600 eukaryotic proteins (for a review, see Aasland et al., 1995). In particular, PHDs are often found in proteins that function in the formation, maintenance, or regulation of chromatin structure, and are thought to function as protein interaction domains in this context. For example, the PHD fingers of human MLL can both mediate homodimerization and bind to Cyp33, a nuclear cyclophilin (Fair et al., 2001). Recently, a region of the corepressor KAP-1 incorporating its tandem PHD finger and bromodomain was found to repress transcription and be essential for the interaction of KAP-1 with Mi-2α (Schultz et al., 2001). In addition, several PHDs have been implicated in the ubiquitination pathway, and in fact appear to function as E3 ubiquitin ligases (Boname and Stevenson, 2001; Coscoy and Ganem, 2003; Mansouri et al., 2003). One such example is the PHD domain of the kinase MEKK1, which has been shown to exhibit E3 ubiquitin ligase activity toward the kinase ERK2 both in vitro and in vivo (Lu et al., 2002).

The abundance and diverse sequences of PHD motifs found in nature suggest that this domain is able to mediate many different interactions and may fulfill a variety of cellular roles. The PHD motifs identified to date are between 50 and 100 residues in length and share a consensus sequence, where the 8 underlined residues ligate two zinc ions (Capilli et al., 2001; Pascual et al., 2000). The sequence can be considered to comprise four sequential pairs of zinc ligands, separated by three loops (X9–21, X4–5, and X12–46; or L1, L2, and L3, respectively). Alignment of PHD sequences (Aasland et al., 1995) from different proteins shows that little conservation exists in the L1 and L3 sequences. This variation suggests that the L1 and L3 regions may be responsible for specifying the binding properties of individual PHDs (Figure 1; Capilli et al., 2001; Pascual et al., 2000).

We have therefore asked whether these regions may be manipulated to produce PHDs with new binding specificities. In order to address this question, we first determined the solution structure of the second PHD finger from Mi2β (Mi2β–P2), a protein that forms part of the transcription silencing NuRD (nucleosome remodeling and histone deacetylation) complex (Ahringer, 2000). The fold of Mi2β–P2 consists of a cross-brace ligation topology that binds two zinc atoms, in agreement with the two other reported PHD structures. The L1 and L3 loops display a higher degree of flexibility than the remainder of the structure, consistent with their sequence variability. We used this information to design several mutants that probed the robustness of the PHD fold by introducing mutations and extensions in the L1 and L3 loops. We show here that all of these mutants are capable of binding Zn(II) and appear to form native-like structures, judging from 1H NMR spectra. Using this strategy, we have also successfully introduced a novel binding function into Mi2β–P2. The 5 additional residues PVDSLs were inserted into L3, thereby creating a derivative that binds specifically to the transcriptional corepressor.
CtBP2 both in vitro and in cellular assays. These results highlight the robustness and adaptability of zinc binding domains and pave the way for their use as protein design scaffolds.

**Results**

**Mi2β-P2 Forms Structure in a Zinc-Dependent Manner**

PHDs from the corepressor KAP-1 and the human Williams-Beuren syndrome transcription factor (WSTF) have previously been shown to ligate two zinc atoms through seven conserved cysteines and one histidine (Capili et al., 2001; Pascual et al., 2000). In order to ascertain whether Mi2β-P2 (residues 446–501; Figure 1) also ligates zinc, we purified the domain to homogeneity using GSH affinity and ion exchange chromatography, diazylated the protein into a zinc-free buffer, and subjected the sample to atomic absorption spectroscopy (AAS). A ratio of 2.3 ± 0.2 zinc atoms per protein molecule was obtained, in agreement with previous data. In previous studies of the PHDs from KAP-1 and WSTF, the addition of the metal chelating agent EDTA to the folded PHD resulted in a complete loss of secondary structure (Capili et al., 2001; Pascual et al., 2000). In contrast, it appears that the affinity of Mi2β-P2 for Zn(II) is substantially higher; the addition of EDTA up to 1 mM did not affect the fold of the protein, judging from circular dichroism spectra (data not shown). One-dimensional 1H NMR spectra of samples obtained with or without 0.7 Å apart. The 8 metal binding residues can be considered as four pairs: C12 with C15, C24 with C27, H32 with C35, and C50 with C53. In this arrangement, the second site, both C23 and C24 are possible fourth ligands. In order to determine the identity of the fourth ligand, we incorporated distance restraints that defined either C23 or C24 as the fourth zinc ligand. Structures calculated on the assumption that C24 was involved in metal coordination not only exhibited a lower overall target function, but also contained far fewer NOE violations around the second zinc site. Subsequent calculations were carried out using the ARIA protocol and these included two zinc atoms as well as additional constraints defining tetrahedral coordination. The remaining ambiguous NOEs were introduced iteratively in ARIA in an automated manner. The 20 lowest energy structures from the final ARIA calculations were chosen to represent the solution structure of Mi2β-P2 (Figure 2A). Structural statistics for the ensemble are given in Table 1.

The Three-Dimensional Structure of Mi2β-P2

Mi2β-P2 adopts a compact globular fold that incorporates two zinc atoms in a cross-braced manner (Figure 2B). The two zinc ions are on average located 15.0 ± 0.7 Å apart. The 8 metal binding residues can be considered as four pairs: C12 with C15, C24 with C27, H32 with C35, and C50 with C53. In this arrangement, the first and third pairs form the first zinc coordination site while the second and fourth pairs form the second. The first pair is contained within an ordered but irregular 14-residue loop that continues into the first strand of a short 8-helical segment (P51–C53) is also observed. Apart

**Structure Determination**

Full spectral assignment and the solution structure of Mi2β-P2 were determined using mostly homonuclear NMR methods. The quality of TOCSY and NOESY spectra was high and, following iterative cycles of manual NOE assignment and structure calculations in DYANA (Guntert et al., 1997), a preliminary global fold of the domain was determined. No reference to metal ligation was included in the calculations at this stage, so that the residues involved in zinc binding could be identified in an unbiased manner. Preliminary structures revealed a cross-brace ligation scheme (Grishin, 2001) and it was evident that the thiol groups from C12, C15, and C35, together with the N36–L49 proton of H32, composed the first zinc coordination sphere (see Figure 1 for numbering scheme). This was further confirmed by measuring the chemical shifts of the histidine side chains in a 2D 1H-15N HSQC spectrum. While the preliminary structures indicated that C27, C50, and C53 formed part of the second site, both C23 and C24 were possible fourth ligands. In order to determine the identity of the fourth ligand, we incorporated distance restraints that defined either C23 or C24 as the fourth zinc ligand. Structures calculated on the assumption that C24 was involved in metal coordination not only exhibited a lower overall target function, but also contained far fewer NOE violations around the second zinc site. Subsequent calculations were carried out using the ARIA protocol and these included two zinc atoms as well as additional constraints defining tetrahedral coordination. The remaining ambiguous NOEs were introduced iteratively in ARIA in an automated manner. The 20 lowest energy structures from the final ARIA calculations were chosen to represent the solution structure of Mi2β-P2 (Figure 2A). Structural statistics for the ensemble are given in Table 1.

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from the short β sheet and α helix, there are no other regular elements of secondary structure and the PHD is largely made up of extended and irregular loops. The overlay of the 20 lowest energy structures (Figure 2A) identifies two flexible regions, K16–E20 and P44–L49, within the otherwise well-defined structure (rmsd = 0.37 Å over residues 9–15, 21–43, and 50–53 for backbone atoms Cα, C′, and N). In contrast, the two zinc binding sites are very well ordered. Numerous hydrogen bonds and hydrophobic interactions appear to stabilize the structure, especially across the β sheet. Y31 and W48 form the center of the hydrophobic core, packing against L21, L22, S29, S30, H32, and L36.

Comparison with the PHDs from WSTF and KAP-1

Recently, the solution structures of two PHDs from other proteins, the corepressor KAP-1 (Capilli et al., 2001) and WSTF (Pascual et al., 2000), have been reported. Pairwise sequence alignments of Mi2β-P2 with these PHDs show that they share 43%–53% conserved residues (excluding zinc-ligating residues). Apart from the L1 loop (which varies in length from 8 to 11 residues), all interring sequences between the conserved cysteines and histidine are the same length for the three PHDs. An overlay of the backbone of the structured regions of Mi2β-P2 (residues 9–15, 24–43, and 48–53) with WSTF-PHD and KAP-1-PHD is shown in Figures 2C and 2D, respectively. The rmsds for overlays of ordered backbone atoms (N, Cα, and C′) is 2.5 Å for Mi2β-P2/KAP-1 and 3.6 Å for Mi2β-P2/WSTF. Not surprisingly, the presence of 3 extra residues in the first loop in WSTF-PHD results in a significantly worse alignment with Mi2β-P2. In particular, the orientation of the L1 loop is substantially shifted. In all cases, the interzinc distance is 14–15 Å, while the rmsd of backbone atoms of the hydrophobic β hairpin core and zinc sites is 0.9 Å. A close examination of the three structures reveals many side chain hydrophobic contacts present in Mi2β-P2 are also conserved in KAP-1-PHD and WSTF-PHD, including contacts involving W48, Y31 (replaced with phenylalanine), L21, and L22 (replaced with valine and isoleucine, respectively). These hydrophobic residues, together with the zinc-ligating residues that are stabilized by the zinc atoms, provide PHDs a common structural scaffold.

In L1-CHD3 and L1-WSTF, we exchanged L1 for the corresponding regions from the second PHD domain in C. elegans CHD3 (Woodage et al., 1997) and human WSTF, respectively. L1-X3 and L1-X6 contained expanded versions of L1, with three and six extra amino acids inserted in the center of the loop, respectively. An analogous set of mutants was made in L3 with an additional mutant L3-X9 that had nine amino acids inserted in the center of L3. Each of the mutants was overexpressed and purified as described for the wild-type Mi2β-P2 domain. Atomic absorption spectrometry revealed that all mutants bound two molar equivalents of Zn(II) (data not shown), and far-UV circular dichroism spectra of the mutants indicate that all nine have similar secondary structure content to the wild-type domain (data not shown). One-dimensional 1H NMR spectra (Figure 4) indicate that all mutants are folded and monomeric; both chemical shift dispersion and line widths are comparable to the wild-type spectrum. Further, the positions of highly shifted signals are essentially preserved, suggesting that there has been little perturbation of the overall fold. One change that can be seen in the spectra of the L3 extension mutants (L3-X3, L3-X6, and L3-X9) is the presence of some additional signals with intensities ~20%–25% of the main signals (indicated with arrows in Figure 4). The chemical shifts and line widths of these additional signals indicate that they correspond to an additional conformation that is both structured and monomeric, although we did not characterize this form in more detail.

In order to assess whether the mutants adopted the same fold as the wild-type Mi2β-P2, we determined the solution structure of the substitution mutant L3-WSTF. Figures 5A and 5B show that the structure is indeed preserved in the mutant. L3-WSTF overlays with Mi2β-P2 with an rmsd (over backbone Cα, N, and C′ atoms) of 1.75 Å. The backbones of Mi2β-P2 and L3-WSTF overlap well with each other except for a small difference observed at residues 20–22 and 43–46, which correspond to the two flexible regions.

Introducing a New Function into Mi2β-P2

Given this result, we sought to introduce a new function into the Mi2β-P2 domain. Many transcriptional repressors contain the motif PXDLS, where X is any residue. It is known that the transcriptional corepressor CtBP2 binds to this motif (Turner and Crossley, 2001) and repression is then brought about by mechanisms that are not well understood. In order to see whether we could create a novel CtBP2 binding domain based on Mi2β-P2, we created a tenth mutant, L3-PVDSL (Figure 3), in which the sequence PVDSL was inserted into L3 between residues 45 and 46. L3-PVDSL gives a 1D 1H NMR spectrum that is very similar to those of L3-X6 and L3-X9, indicating that it is able to form a well-ordered structure and that it is probably monomeric. A GST pull-down assay revealed that GST-L3-PVDSL was able to specifically pull murine CtBP2 out of a bacterial cell lysate, while neither wild-type Mi2β-P2 nor L3-X6 exhibited significant binding (Figure 6A). To confirm the specificity of this interaction, we used a yeast two-hybrid assay in which L3-PVDSL and CtBP2 were fused to the activation
Table 1. Structural Statistics for Mi2-P2 and L3-WSTF

<table>
<thead>
<tr>
<th>Experimental Input</th>
<th>Mi2-P2</th>
<th>L3-WSTF</th>
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</thead>
<tbody>
<tr>
<td>Total NOE restraints</td>
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<td>Total unambiguous restraints</td>
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<tr>
<td>Total ambiguous restraints</td>
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<td>23</td>
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<td>10</td>
<td>11</td>
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<tr>
<td>PROCHECK statistics</td>
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<tr>
<td>Residues in allowed regions</td>
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<td>2.4%</td>
</tr>
<tr>
<td>Residues in disallowed regions</td>
<td>0.0%</td>
<td>0.1%</td>
</tr>
<tr>
<td>RMSd of Backbone Atoms</td>
<td>0.51 ± 0.09</td>
<td>0.91 ± 0.13</td>
</tr>
<tr>
<td>RMSd of All Heavy Atoms</td>
<td>0.92 ± 0.11</td>
<td>1.53 ± 0.20</td>
</tr>
<tr>
<td>Bond lengths</td>
<td>0.0049 ± 0.0006 Å</td>
<td>0.0050 ± 0.0006 Å</td>
</tr>
<tr>
<td>Bond angles</td>
<td>0.507 ± 0.039Å</td>
<td>0.540 ± 0.065Å</td>
</tr>
</tbody>
</table>

Discussion

One of the striking aspects of zinc binding domains is that they have evolved to make contacts with a wide range of different substrates, including single-stranded, double-stranded (Pavletich and Pabo, 1991), and tetraplex (Choo and Klug, 1994) DNA, RNA, other proteins (Mackay and Crossley, 1998), and even lipids (Gaulier et al., 1998). In many cases, proteins may contain multiple zinc fingers, which may act either to alter DNA binding specificity, to allow the crosslinking of multiple protein partners, or both. It has also been shown that, for at least two separate classes of zinc binding domains (Michael et al., 1992; Sharpe et al., 2002), ~70%–80% of the amino acids can be mutated to alanine without disrupting the structure. This modular nature and tolerance to mutation has been successfully exploited in a large number of studies that have used phage display methods combined with structural information to engineer zinc finger arrays that recognize specific DNA sequences (reviewed in Choo and Isalan, 2000). The properties of zinc fingers make them good candidates for intracellular protein scaffolds: they are small, stable, not reliant on disulfide bonds, and have been used successfully in phage display screens, and the variety of structural motifs available increases the opportunity of selecting a suitable scaffold for a given target.

From the data available, it appears that PHDs most likely act as binding motifs that contact proteins and/or other biomolecules. It has been suggested that the specificity for binding different targets may be imparted by the loop regions L1 and L3 (Capili et al., 2001; Pascual et al., 2000), and it was this suggestion that prompted us to investigate whether L1 and L3 could serve as sites for generating novel binding proteins. After determining the solution conformation of Mi2-P2, we were able to generate a family of mutants in which L1 and L3 were either expanded or substituted. Further, we successfully introduced a novel binding function into Mi2-P2, turning it into a CtBP2 binding protein (Michael et al., 1992; Sharpe et al., 2002).

This work demonstrates that the structural plasticity of the L1 and L3 regions can be exploited to introduce new binding functions into the domain. The existence of a wide range of L1 and L3 sequences in existing PHDs, together with the diversity of proteins in which PHDs are found, points to the likelihood that PHD fingers might constitute suitable scaffolds for mutation by
expansion and/or substitution to create a wide range of new binding proteins. As such, PHD fingers offer several advantages over other described systems as protein scaffolds. The small size of PHDs offers the possibility of transporting designed versions into cells, while their structural stability should ensure both that a wide range of mutational challenges (which will be necessary to create functional diversity) can be tolerated and that they will be relatively resistant to degradation. This is generally not the case with small peptide aptamers, where small sequence changes are more likely to cause conformational changes in the scaffold. Further, the presence of two mutatable loops opens up the possibility of bifunctional variants, where one loop may bind a target while the second recruits other cellular machinery (e.g., to promote ubiquitination of the target). Finally, the domain does not contain any redox active metal centers (c.f. thioredoxin; Colas et al., 1996) of disulfide bonds (c.f. antibodies), improving its suitability for intracellular use.

Our demonstration of the robust and flexible nature of the PHD fold should pave the way for more extensive manipulations directed toward the development of tailored binding proteins based on zinc binding scaffolds. Results such as these may bode well for our prospects of manipulating gene expression in vivo, with tailored reagents, ultimately for experimental or therapeutic purposes.

Biological Implications
Antibodies can be readily manipulated to recognize cell surface proteins and other extracellular targets. However, progress in creating specific reagents that can target intracellular species has been substantially slower. A protein “scaffold,” comparable to the antibody constant region, which fulfills the requirements for small size and high stability in an intracellular environment and carries variable regions with a high tolerance for mutation would be very valuable in this regard. Our work has demonstrated that the PHD zinc finger possesses these desirable properties. It is small, has a well-structured core that contains two zinc ions, and carries two variable and flexible loop regions. We have shown that these loops tolerate both substitution and extension mutations without compromising the overall fold of the domain. Further, specific binding functions can be introduced into the loops to create novel binding proteins that are functional both in vitro and within eukaryotic cells. These results indicate that the PHD domain represents a promising candidate for a good scaffold for protein design or redesign.

Experimental Procedures
Preparation of the Mi2β-P2 Domain
The Mi2β-P2 domain was cloned by PCR amplification from a CDNA template. The DNA fragment was ligated into the pGEX-2P expression vector (a modified pGEX-2T vector that contains a PreScission cleavage site) and expressed in E. coli BL21 cells. Glutathione-S-transferase (GST)-tagged protein was expressed at 37°C in either Luria broth or in minimal medium containing 15NH4Cl as the sole nitrogen source. Pellets were lysed in a buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 1.4 mM DTT, and 1 mM ZnSO4. The fusion protein was then purified by glutathione (GSH) affinity chromatography, cleaved with PreScission protease (for 24–72 hr), and further purified using anion exchange chromatography (Mono Q HR 10/10, Pharmacia; pH 7.5). The domain comprised five N-terminal amino acids (GPLGL) derived from the PreScission cleavage site fused to amino acids 446–501 of Mi2β, and its identity was confirmed using electrospray mass spectrometry (M theor 6680.8 Da; M obs 6681.3 ± 0.6 Da).

Mutants were prepared using overlap PCR and were overexpressed and purified in the same manner as the wild-type protein.

Atomic Absorption Spectrometry
Samples were diluted and dialyzed in 10 mM sodium phosphate and 1 mM DTT (pH 7.5) to a final concentration of 3 μM. Measurements were performed on a Varian Spectra AA20plus atomic absorption spectrometer at 213.9 nm using solutions of ZnNO3 as the calibration standards. All measurements were repeated five times.

Circular Dichroism Spectropolarimetry
Samples were prepared by buffer exchanging samples into a buffer containing 10 mM sodium phosphate and 1 mM DTT (pH 7.5) to...
Figure 4. One-Dimensional $^1$H NMR Spectra of Mi2$\beta$-P2 and Mutants

Spectra were recorded at 298 K and pH 7.5. Additional signals corresponding to a second conformer are marked with arrows and the DSS used as a chemical shift reference is marked with an asterisk.

NMR Spectroscopy

For NMR experiments, the purified Mi2$\beta$-P2 and L3-WSTF domains were centrifugally concentrated (in a buffer containing 150 mM NaCl, 10 mM sodium phosphate, and 1 mM DTT [pH 7.5]) to 1.0–1.5 mM for unlabeled Mi2$\beta$-P2 and L3-WSTF or 100 $\mu$M for $^{15}$N-labeled Mi2$\beta$-P2 protein. The final NMR sample also contained 20 $\mu$M 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as a chemical shift reference and 5% (v/v) D$_2$O. Spectra were recorded at 25°C on a Bruker DRX600 spectrometer. All homonuclear two-dimensional data were collected and analyzed as described (Kowalski et al., 1999; Liew et al., 2000). TOCSY spectra were recorded with mixing times of 35 and 70 ms, and NOESY spectra were recorded with mixing times.
Figure 5. Solution Structures of L3-WSTF and Overlay with Mi2j-P2

(A) Ensemble of the best 20 structures of L3-WSTF. Structures are superimposed over the backbone atoms (C\(^\beta\), C\(^\gamma\), N) of residues 9–43 and 48–53 (residues 1–7 and 55–61, which are unstructured, are omitted for clarity). The zinc-chelating side chains are shown in yellow and green, and the zinc atom is shown in magenta. The L1 and L3 loops are shown in blue and red, respectively. Structures are shown as wall-eyed stereo images.

(B) Overlay of ribbon diagrams of the lowest energy structures of L3-WSTF and Mi2j-P2 showing elements of secondary structure as recognized in MOLMOL.

of 50, 200, and 250 ms. The protonation state of the histidine side chains was carried out as described (Pelton et al., 1992). For stereo-specific assignments and \(\phi\) angle restraints, 2D NOESY and TOCSY spectra (with \(T_\text{m}'\)s of 50 and 35 ms, respectively) were analyzed. \(^3J_{\text{HN,H}}\) coupling constants were obtained using the program INFIT (Szyperski et al., 1992). All NMR data were processed using XWINNMR (Bruker) and analyzed with the program XEASY (Bartels et al., 1995).

Structure Calculations

NOE-derived distance restraints were obtained from the 2D \(^1H\)-NOESY spectra and calibrated using the CALIBA module of DYANA (Guntert et al., 1997). Dihedral angle restraints for \(\phi\) angles were set to \(-60 \pm 40^\circ\) for \(^3J_{\text{HN,H}}\) < 6 Hz and \(-120 \pm 40^\circ\) for \(^3J_{\text{HN,H}}\) > 8 Hz. \(\phi\) and \(\chi\) constraints were obtained as described previously (Liew et al., 2000). Three additional \(\psi\) angle constraints were included using methods described in Gagne et al. (1994).
Figure 6. Engineering a New Function into Mi2β-P2

(A) GST pull-down assay showing the binding of murine CtBP2 to L3-PVDLS. Lanes 1 shows 20% input CtBP2 as a bacterial cell lysate. Lanes 2 and 3 contain wild-type Mi2β-P2, lanes 4 and 5 contain L3-PVDLS, and lanes 6 and 7 contain L3-X6. CtBP2 cell lysate was added to lanes 3, 5, and 7. Samples were run on a 15% polyacrylamide gel and stained with Coomassie blue.

(B) Yeast two-hybrid analysis of the L3-PVDLS:CtBP2 interaction. Yeast cotransformed with the plasmids shown grew on SD-L-T media (left panel). These transformants were patched onto SD-L-T-H media (right panel). Growth after 60 hr at 30°C is shown.

Structure calculations were performed initially in DYANA (Guntert et al., 1997) and later in CNS (Brunger et al., 1998) using ARIA (Nilges, 1995; Nilges et al., 1997). Zinc coordination (Neuhaus et al., 1992) was incorporated in the ARIA calculations, which were carried out using a standard protocol (Nilges, 1995; Nilges et al., 1997). The final assignments made by ARIA were checked and corrected manually where necessary. Overall, 5 (for Mi2β-P2; 6 for L3-WSTF) NOEs for which no consistent assignment could be determined were excluded from the final calculations.

Calculations were carried out in the simplified all-hydrogen PAR-ALLHDG5.2 force field with nonbonded interactions modeled by PROLSQ force field (Linge and Nilges, 1999); floating chirality assignment (Folmer et al., 1997) was used for all methylene and isopropyl groups for which no stereo-specific assignment could be made. Finally, the 50 lowest energy structures were refined in a 9 Å shell of water molecules (Jorgensen et al., 1983). The 20 conformers with the lowest value of E_in were visualized and analyzed using the programs MOLMOL (Koradi et al., 1996), PROCHECK (Laskowski et al., 1996), and WHATIF (Vriend and Sander, 1993).

GST Pull-Down Assay
Fusion P2 and mutant proteins were purified as described. Small aliquots of GSH-Sepharose beads containing fusion proteins were run on SDS-PAGE for protein concentration estimation, after which the amounts of beads in each sample were adjusted to have roughly the same amount of protein. After washing the samples with a buffer containing 50 mM Tris (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% Triton X-100, and 10% glycerol, 100 μl of soluble cell lysate containing overexpressed CtBP2 was added to the bead
samples and incubated at 4°C for 1 hr. The samples were washed as above six times and run on SDS-PAGE.

**Yeast Two-Hybrid Assay**
The yeast two-hybrid system was performed as described in the manufacturer’s protocol (Clontech). Briefly, the Phd finger of Mi2β with or without the PVDSL insertion was cloned into-frame in the Gal4 activation domain plasmid pGAD10. These plasmids were co-transfected with the Gal4 DNA binding domain fused to mCTBP2 (Tumer and Crossley, 1998) into the yeast strain H7C7, and transformants were selected on Trp/Leu-deficient media (SD-L-T). Colonies were patched onto Trp/Leu/His-deficient media (SD-L-T-H).

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