The PR Domain of the Rb-binding Zinc Finger Protein RIZ1 Is a Protein Binding Interface and Is Related to the SET Domain Functioning in Chromatin-mediated Gene Expression*

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The PR domain, first noted as the PRDI-BF1-RIZ1 homologous region, defines a sub-class of zinc finger genes that appear to function as negative regulators of tumorigenesis. This family includes the MDS1-EVI1 gene inactivated in myeloid leukemia, the PRDI-BF1/BLIMP1 transcription repressor of c-myc involved in driving B-cell differentiation, and the RIZ gene, which encodes proteins capable of binding to the retinoblastoma tumor suppressor protein (Rb). The PR domain of MDS1-EVI1 is disrupted by translocations linked to myeloid leukemia, resulting in the activation of the PR-minus oncogenic product EVI1. Remarkably similar to MDS1-EVI1, RIZ gene also normally produces two protein products of different length, and the smaller protein RIZ2 lacks the PR domain of RIZ1 but is otherwise identical to RIZ1. These observations raise considerable interest to determine the function of PR. We show here that RIZ1 PR domain mediates protein-protein interaction. Recombinant fusion proteins of PR can bind to in vitro translated RIZ1 and RIZ2 proteins. The binding can be disrupted by amino acid substitutions at conserved residues of PR, suggesting that binding is specific. Of the three conserved exons of PR, the first two appear dispensable for binding, whereas the third exon is required. A region in the carboxyl terminus of RIZ proteins was mapped to be necessary and sufficient for PR binding. We also found that the PR domain shares significant sequence identity to the SET domain present in chromosomal proteins that function in modulating gene expression from yeast to mammals. Our data suggest that the PR domain is a derivative of SET domain and may function as protein binding interface in the regulation of chromatin-mediated gene expression.

The Krüppel-like family of zinc finger genes is estimated to consist of hundreds of human genes (1). This family is characterized by the Cys2-His2 zinc fingers and can be further divided into different subsets based on other structural features that include the seven-amino acid “H/C link” between adjacent fingers (2) and several conserved amino-terminal modules (3–7). The PR domain is a newly recognized amino-terminal module and was first noted for the homologous 100-aa region shared between RIZ, which encodes Rb-binding proteins and the PRDI-BF1/BLIMP1 transcription repressor, which promotes B-lymphocyte maturation (8–11). A PR domain was later found in the MDS1-EVI1 myeloid leukemia gene (12). The PR domain is evolutionally conserved, and PR-containing open reading frames were found in the Caenorhabditis elegans and Drosophila genome or EST data bases.2

RIZ gene normally produces two different products, RIZ1 and RIZ2, which are both widely expressed (13). An internal promoter generates RIZ2, which is identical to RIZ1, except that it lacks the RIZ1 PR domain. This is remarkably similar to MDS1-EVI1. An internal promoter within MDS1-EVI1 gene generates the EVI1 myeloid-transforming gene product, which lacks PR but is otherwise identical to MDS1-EVI1 (14). The PR domain of MDS1-EVI1 is a common target of inactivation by viral insertions and chromosomal translocations in leukemogenesis (15–17), suggesting that the PR domain plays an important negative role in tumorigenesis. Consistently, other PR-containing gene products also have characteristics of a negative regulator of tumorigenesis. The BLIMP1 gene maps to a tumor suppressor locus 6q21 (18) and is a transcriptional repressor of the c-myc oncogene, which is commonly overexpressed in a variety of human cancers (19). Forced expression of BLIMP1 causes differentiation or apoptosis, depending on cell types (11, 19). A tumor suppressor role for RIZ is suggested by its chromosomal location at the distal short arm of human chromosome 1 next to the marker D1S228 on 1p36.23 (20, 21), which commonly undergoes deletions, rearrangements, or loss of heterozygosity in a broad spectrum of human tumors, including those of breast (22, 23), liver (24), colon (25), and neurocristic tissues (26, 27). We have recently established that RIZ1, but not RIZ2, expression was commonly decreased or lost in breast cancer, and forced RIZ1 expression caused cell cycle arrest in G0/M and/or apoptosis.3

Recent investigation shows that the PR domain of RIZ1 has neither a transcriptional repressor nor activator function (28), although full-length RIZ gene products function in both transcription activation (21) and repression (28). Both RIZ1 and RIZ2 bind to GC-rich Sp-1-like DNA elements and repress transcription of the simian virus 40 early promoter, but RIZ1 is a more potent repressor than RIZ2, suggesting that the PR domain of RIZ1 modulates transcription (28). MDS1-EVI1 is recently shown to act as a transcriptional activator, whereas EVI1 as a repressor; the activation domain of MDS1-EVI1 maps to the PR domain (47). Although these studies shed light

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1 The abbreviations used are: aa, amino acids; EST, Expressed Sequence Tag; GST, glutathione S-transferase; PCR, polymerase chain reaction.
2 S. Huang, unpublished observation.
3 L. He, J. X. Yu, L. Liu, I. M. Buyse, M.-S. Wang, Q. Yang, A. Nakagawara, G. M. Brodeur, Y. E. Shi, and S. Huang, submitted for publication.
on understanding PR domain function, no biochemical activities have been described for this important domain.

Conserved motifs found in transcription factors generally function in binding to DNA and/or to polypeptides. We have generated purified recombinant fusion proteins of RIZ1 PR domain and used these proteins to examine whether this domain serves as a polypeptide binding interface. We found that PR domain polypeptides interact with in vitro translated RIZ1 or RIZ2 proteins. The binding appears to be specific, as it can be disrupted by specific mutations in the PR domain. A region in the carboxyl terminus of RIZ proteins was mapped to be necessary and sufficient for PR binding. The results demonstrate that the PR domain of RIZ1 mediates protein-protein interaction. We also found that the PR domain shares significant sequence identity to the SET domain present in chromosomal proteins that function in modulating gene expression from yeast to mammals. Our data suggest that the PR domain is a derivative of SET domain and may function as the protein binding interface in the regulation of chromatin-mediated gene expression.

MATERIALS AND METHODS

**Plasmid Constructions**—The RIZ1 PR domain was expressed as a GST fusion protein using the pGEX-KG vector as described previously (8). A HincII-Stul PR domain fragment corresponding to residues 37–214 of rat RIZ1 cDNA was subcloned into the blunted Ncol site in pGEX-KG to generate pGST-RIZ1-PRB. To remove non-PR domain sequences in pGST-RIZ1-PRB, a PCR fragment was amplified using pGST-RIZ1-PRB as a template with primer GEX2 (5′-CTGTTGCT- GGTGATCC-3′) and primer RP197 (5′-GGAAGCTTACAGCTG- CTTCTACAGTCTCCCTGCGTCTT-3′). The PCR fragment was cloned into the EcoRI and HindIII sites in pGEX-KG to generate pGST-RIZ1-PR (aa 37–172).

Amino acid substitutions were introduced into the PR domain to generate pGST-RIZ1-L104A using the Quick-Change mutagenesis kit from Stratagene. The two Leu at position 104–105 in Fig. 1 were changed to Ala. The oligonucleotides used were 5′-CTCGGAGGGAGGAGGCCGCCGTTGCTGTAACAGC-3′ and 5′-CTTCTGGAGCGCCCGACAGCCCGCGGCTCCTCGCCAGG-3′.

For deletion of coding exon 5 or block C of PR domain, an SpeI linker (5′-CTAGACTAGTCTAG-3′) was inserted into the SacI site in the pGST-RIZ1-PR construct to generate pGST-RIZ1-PRΔC (express aa 37–135). Coding exon 4 or block B (aa 77–127) deletion construct pGST- RIZ1-PRΔB was generated by replacing the PR domain insert in GST-RIZ1-PR with an reverse transcription-PCR fragment lacking exon 4. The RNA used for reverse transcription-PCR was prepared from brain tissue of a mutant mouse line homozygous for an in-frame deletion of exon 4. The primers used were RP244 (5′-TATCATGGAAAGACTGGATTGTGTCCTGACCTAAC-3′) and RP197. Coding exon 3 or block A (aa 37–76) deletion construct pGST-RIZ1-PRΔA was generated by cloning a PCR fragment of pGST-RIZ1-PR into the XhoI and HindIII sites in pGEX-KG to generate pGST-RIZ1-PR (aa 37–172).

Plasmids pGR1zR, pCMVRIZ, and pGR1zRKK for producing RIZ1 and RIZ2 proteins by in vitro translation have been described previously (13). Because yield of in vitro translation products produced by the TNT lysate (Promega) was lower than expected, the mixture was then incubated with glutathione-Sepharose 4B. Binding and washing buffers are the same: 50 mM Hepes, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM of dithiothreitol, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride. After washing 4×, proteins bound to the beads were analyzed by SDS gel electrophoresis. The electrophoretic patterns were visualized by staining with Coomassie Blue or autoradiography.

**Data Base Searching**—The peptide sequence of the PR domain of RIZ1 was used as a query to search the nonredundant protein data base of NCBI (National Center for Biotechnology Information). The position-specific-iterated BLAST (PSI-BLAST) program for motif-style searching was used, which is more sensitive to distant relationships (30).

RESULTS

**The PR Domain of RIZ1 Functions in Protein Interaction**—RIZ gene contains eight coding exons, and the seventh coding exon encodes the majority of RIZ1 protein (85.6%) (Fig. 1A). The PR domain is encoded by three small exons, coding exons 3–5. Each exon in PR contains a conserved block of residues, designated A, B, or C in Fig. 1B. The PR domain is located in the amino-terminal 200-amino acid region of RIZ1 consisting of coding exons 1–6 (Fig. 1A). This entire sequence is absent in RIZ2 protein (Fig. 1A). This region is thus expected to confer RIZ1 specific functions. To help study activities of this amino-terminal region and in particular the PR domain, two different GST fusion proteins were produced in E. coli and purified (Fig. 2). GST-RIZ1-PR protein (rat RIZ1 residues 37–214) contains most of the amino-terminal 200-amino acid sequence consisting of part of coding exon 2 and entire coding exons 3–6. This protein could be used to examine the function of the amino-terminal region including PR and its proximal sequences. GST-RIZ1-PR protein (residues 37–172) contains the minimal PR domain sequence as defined by homology with other PR domain genes and consists of part of coding exon 2 and entire coding exons 3–5. This protein could be used to examine the function of PR only.

The conserved motifs of many transcription factors are known to mediate homo-oligomeric interactions. We therefore examined whether PR could interact with RIZ1 protein. GST fusion proteins as shown in Fig. 2B were bound to glutathione-Sepharose 4B beads. The protein-loaded beads were then incubated with radiolabeled in vitro translated RIZ1 protein. Binding (approximately 10% of input RIZ1 protein was bound) was observed for either GST-RIZ1-PR- or GST-RIZ1-PRB-loaded beads (Fig. 3A, compare lane 1 to lane 3 or 4), whereas no binding was found for the GST protein-loaded beads (Fig. 3A, produced to express aa 900 to 1272 by cloning the BstEII to BamHI fragment into pcdNA3. Plasmid pGR1zRANBam was generated to express the carboxyl-terminal region (aa 1272–1706) by in-frame deletion of the amino-terminal region from Ncol to BamHI in pGR1zR. The following enzymes were used to digest the construct pGR1zRANBam to generate carboxyl-terminal truncation mutants for in vitro translation: EcoRI, ApoI, and NsiI.

For constructing GST fusion proteins of the carboxyl-terminal region pGST-hRIZ1-1514–1680, human RIZ1 cDNA was PCR-amplified using primers RP252 (5′-GCATTGATGAGCCGGCAGCGGATGG-3′) and RP253 (5′-TACGAGGCTTCTAGTGAAGGCTGCTCTTG-3′). The PCR fragment was cloned into the EcoRI and HindIII sites in pGEX-KG. This region of human RIZ1 protein corresponds to 1500–1668 of rat RIZ1 protein.

Expression and Purification of Recombinant Protein—Expression of the recombinant proteins from plasmids bearing log-phase Escherichia coli XL1-blue cells was induced with 0.4 μM isopropyl-β-D-thiogalactopyranoside, and bacterial culture proceeded for 3 h at 30 °C. Protein extract was made, and recombinant GST fusion protein was purified as described (29).

GST Pull-down Assay—Glutathione-conjugated Sepharose 4B (Amersham Pharmacia Biotech) was used to purify various GST fusion proteins from E. coli cell extracts. In some experiments, the protein-loaded beads were then incubated with in vitro translated products. Alternatively, the purified proteins or bacterial cell extracts were first mixed with in vitro translation products produced by the TNT lysate (Promega). The mixture was then incubated with glutathione-Sepharose 4B. Binding and washing buffers are the same: 50 mM Hepes, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM of dithiothreitol, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride. After washing 4×, proteins bound to the beads were analyzed by SDS gel electrophoresis. The electrophoretic patterns were visualized by staining with Coomassie Blue or autoradiography.
These results suggest that the amino-terminal 214-amino acid region of RIZ1 participates in protein-protein interactions between RIZ1 molecules. Binding to RIZ1 was demonstrated even for the minimal PR domain. Similarly, in vitro translated RIZ2 product also bound to GST-RIZ1-PR- or -PRB-loaded beads (Fig. 3A, lanes 5–8). The binding of PR to RIZ2 appeared to be comparable with binding to RIZ1, indicating that PR homo-oligomeric interaction, if there is any, is not essential for homo-oligomeric binding of RIZ proteins. To confirm that the PR motif does not strongly interact with itself, in vitro translated, radiolabeled PR domain product (residues 37–214) was examined for binding to GST-RIZ1-PR protein. Approximately 1% of input protein was bound, which is significantly weaker than PR-RIZ1 or PR-RIZ2 interactions (Fig. 3B). The results suggest a weak homo-oligomeric interaction of PR. However, such weak interaction is probably not responsible for the strong binding observed in Fig. 2.

To better characterize the observed protein binding activity of PR, deletion and missense mutations were introduced into the GST-RIZ1-PR protein construct. To assess the role of each conserved exon in PR activity, mutants deleting each exon were constructed and expressed in E. coli. To assess the binding interactions with GST-RIZ1-PR, GST-RIZ1-PRB, and GST proteins are shown in lanes 2–4, respectively.

FIG. 3. Binding of PR domain protein to RIZ1 and RIZ2 proteins. A, bacterial extracts expressing GST or GST-RIZ1-PR proteins as indicated were mixed with glutathione-Sepharose 4B beads. The protein loaded beads were then mixed with in vitro translated, radiolabeled RIZ1 or RIZ2 protein. After washing, bound proteins were analyzed by SDS gel electrophoresis followed by autoradiography. In vitro translated RIZ1 and RIZ2 proteins (about 10% of input) are shown in lanes 1 and 5. RIZ proteins bound by GST are shown in lanes 2 and 6, by GST-RIZ1-PR in lanes 3 and 7, and by GST-RIZ1-PRB in lanes 4 and 8. B, PR domain weakly interacts with itself. In vitro translated and radiolabeled PR protein as shown in lane 1 (10% of input) was incubated with GST-RIZ1-PR protein-loaded beads. Proteins bound by GST-RIZ1-PR, GST-RIZ1-PRB, and GST proteins are shown in lanes 2–4, respectively.

PR Domain Mediates Protein-Protein Interaction
specificity and the role of some of the conserved amino acid residues, amino acid substitutions were introduced into conserved positions in exon 5, changing two Leu residues at position 104–105 to Ala (PR-L104A/L105A). These mutant proteins as shown in Fig. 4A were used for binding to in vitro translated RIZ1 protein. As shown in Fig. 4B, deletion of exon 5 disrupted binding (GST-RIZ1-PRdelC), but deletion of exon 3 and 4 did not significantly affect binding (GST-RIZ1-PRdelA and -delB). The mutant protein with substitutions of leucines for alanines at residues 104–105 also disrupted binding. These results suggested that the region from residues 1489 to 1706 (p3RIZrBB) did not affect binding, but deletion of residues 1489–1706 (p3RIZrD) did not affect binding, but deletion of residues 1489–1680 (p3RIZrDNBam) did not affect binding, but deletion of residues 1489–1680 (p3RIZrDNBam) did not affect binding, but deletion of residues 1489–1680 (p3RIZrDNBam) did not affect binding, but deletion of residues 1489–1680 (p3RIZrDNBam) did not affect binding, but deletion of residues 1489–1680 (p3RIZrDNBam) disrupted binding.

To further map the PR binding motif of RIZ proteins, truncation mutants of RIZ1 were generated by in vitro transcription and translation of various mutant constructs (Fig. 5A). Truncated RIZ proteins were examined for binding to GST-RIZ1-PR protein. The carboxyl-terminal half (residues 900–1706, p3RIZrΔNB) strongly bound to PR (about 10% of input RIZ was bound), whereas the amino-terminal half showed only weak binding (approximately 1% of input RIZ) (Fig. 5B). The weak binding of PR to the amino-terminal half of RIZ1 is consistent with the weak PR-homo-oligomeric interactions already described and presented in Fig. 3B. Once the PR binding motif was indicated in the carboxyl-terminal half of RIZ1, deletion mutants of p3RIZrΔNB were generated by in vitro transcription and translation. A fragment encompassing residues 900 to 1272 (construct p3RIZrBB) did not bind to GST-PR, but a fragment encoding residues 1272 to the end codon 1706 (construct p3RIZrΔNBam) retained full PR binding activity. This result narrows the PR binding motif to within the carboxyl-terminal 434-amino acid region (aa 1272–1706) (Fig. 5C).

The PR Domain Is Related to the SET Domain of Chromosomal Proteins—To better understand the biological functions of PR domain, we searched the NCBI data base for protein sequences that may share similarity to the PR domain. PSI-BLAST uses an initial BLAST run to generate a gapped multiple alignment. It then constructs from this alignment a
position-specific score matrix, which is employed as a “query” in a subsequent BLAST search. This process can be repeated multiple times to hunt for homologous sequences that would not have been retrieved by the original BLAST algorithm.

We used the PR domain peptide sequence of RIZ1 as the query and performed PSI-BLAST searches of the nonredundant protein data base of NCBI. All known PR genes as well as two C. elegans open reading frames (F25D7 and T21B10) were identified with E values below $5 \times 10^{-10}$, demonstrating the power of the PSI-BLAST program. The next level of significant matches were found for the SET domain present in G9a, ASH1, Su(Var)3-9, and SET1 proteins with E values ranging from 0.003 to 1.9. The SET domain is a 130-amino acid, evolutionarily conserved sequence motif present in chromosomal proteins that function in modulating gene activities from yeast to mammals (31, 32). It is important to note that the shared residues between PR and SET are also among the most conserved residues in each domain, suggesting they may share a common function (Fig. 7). One of the two conserved Leu residues required for RIZ1 PR protein binding activity as shown in Fig. 4 is also conserved in the SET domain.

Despite the similarity between PR and SET, which is typically ~20% identical in amino acids, they are clearly distinc- tive, because identities among PR genes are typically ~45% and among SET genes, ~50%. Also, SET domains are primarily found at the carboxyl termini of proteins, whereas PR domains are mostly located at the amino termini. PR domains are primarily associated with zinc finger proteins, whereas SET domains have been found in a variety of proteins containing various motifs, including the chromo domain, AT hooks, zinc finger, PHD fingers, and GTP binding motifs (32). Finally, PR domain is not found in yeast genome and is likely a derivative of SET domain in evolution.

DISCUSSION

RIZ1 is identical to RIZ2, except that it has an extra 200 residues at the amino terminus. These extra residues may confer specific function to RIZ1 protein. The PR domain represents the major functional motif within the RIZ1 amino-terminmal region. We found that at least one function of this region is to serve as a protein binding interface. GST fusion proteins containing most of the RIZ1 amino terminus from 37–214 showed similar activities as the minimal PR motif from residues 37–172, suggesting that the PR domain is the protein binding motif contained in the amino-terminal region. The protein binding function of RIZ1 PR is specific, as evidenced by disruption of function by mutations. It is interesting to note that deletion of coding exon 3 or 4 did not significantly affect PR binding to RIZ proteins, even though both exons encode conserved sequences. In contrast, exon 5 is required, as dele-
tion or point mutation within this exon disrupted binding. The observation suggests a modular nature of PR domain action. It is probable that an individual conserved block of PR may possess independent functional activities that are independent of other blocks. Novel activities may also derive from combinations of these blocks.

The mapping of a PR binding motif to a carboxyl-terminal region of RIZ1 represents the identification of a novel motif in this multidomain protein. This motif appears not well conserved among PR genes, as data base searches did not reveal any proteins with significant similarity. Nevertheless, the observation raises the possibility that PR could be engaged in either intermolecular interactions or intramolecular interactions. Which type of interaction would dominate may depend on other protein-protein interactions. Interaction of PR domain with the carboxyl domain could occur as an intramolecular interaction in full-length RIZ1 but not in RIZ2. Homo-oligomerization between RIZ1 molecules or heter-oligomerization between RIZ1 and RIZ2 could be driven by this binding contact. It is also possible that both PR and the carboxyl-terminal PR binding motif of RIZ1 may interact with other as yet unidentified proteins. Understanding this complex network of interactions in vivo represents an important future goal.

That PR domain is related to the SET domain is a provocative finding. SET domain genes have diverse biological functions related to chromatin structure and play an important role in development, cancer, position-effect-variegation, telomeric and centromeric gene silencing, and possibly in determining chromosome architecture (32). They are multifunctional chromatin regulators with activities in both eu- and heterochromatin. The founding members of the family include three Dro sophila genes: the strongest position-effect-variegation suppressor gene Su(Var)3-9 (31), the polycomb group gene Enhancer of zeste (33), and the trithorax group gene trithorax (34). Other prominent members include the Saccharomyces cerevisiae SET1 gene which affects mating-type switching and telomeric silencing (35), the Schizosaccharomyces pombe Chr4+, which is involved in centromere function (36), and the human trithorax homolog HRX (also called ALL-1 and MLL), which is a breakpoint gene involved in human leukemia (37–39).

The SET domain alone can replace the full-length SET1 protein in mediating telomeric gene silencing, suggesting a direct and important role of SET in heterochromatin regulation (35). However, a DNA or chromatin binding activity for the direct and important role of SET in heterochromatin regulation protein in mediating telomeric gene silencing, suggesting a feature shared by many SET genes that function in heterochromatin-mediated gene silencing. Future studies are expected to generate more similarities in the biochemical and biological functions of these genes, although differences may also be expected since PR and SET are clearly two separate motifs.

That the Rb-binding protein RIZ1 is likely to function in chromatin regulation further implicates a role for Rb in regulating chromatin-mediated gene expression. Other Rb-binding proteins are known to function in chromatin modulation, including human histone deacetylase (42–44) and E2F1, whose Drosophila homologs are enhancers of position-effect-variegation (45, 46).

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Note Added in Proof—After submission of this manuscript, three reports have been published describing SET domain function in protein-protein interactions (Rozenblattrosen, O., Rozovskaia, T., Bu rakovsky, S., Sedkov, Y., Tiliil, S., Blechman, J., Nakamura, T., Croce, C. M., Mazo, A., Canaani, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4152–4157; Cui, X. M., Devivo, I., Slany, R., Miyamoto, A., Firestein, R., Cleary, M. L. (1998) Nat. Genet. 18, 331–337; and Cardoso, C., Timsit, S., Villard, L., Khrestchatisky, M., Fortes, M., Colleaux, L. (1998) Hum. Mol. Genet. 7, 679–684. The SET domain of HRX (ALL1) is shown to interact with INI1 and SNR1 proteins of the SWI/SNF complex involved in chromatin remodeling (Rozenblattrosen et al.) and the polytubulin family of dual specificity protein phosphatases (Cui et al.). The SET domain of EZH2 is shown to bind to the XNP/ATR-X gene product, a member of the SNF2-like family involved in chromatin remodeling (Cardoso et al.).

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PR Domain Mediates Protein-Protein Interaction

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