Modular mutagenesis of human placental ribonuclease inhibitor, a protein with leucine-rich repeats

(angiogenin/gene duplication/protein structure–function relation)

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ABSTRACT Human placental ribonuclease inhibitor (PRI) is a potent protein inhibitor of pancreatic ribonucleases and the homologous blood vessel-inducing protein angiogenin. Although inhibition by PRI occurs with a 1:1 stoichiometry, its primary structure is composed predominantly of seven internal leucine-rich repeats. These internal repeats were systematically deleted either singly or in combination by “modular” mutagenesis. Deletion of repeat units 3 plus 4 or repeat unit 6 results in mutants that both bind to and inhibit ribonuclease A. Therefore, the angiogenin/ribonuclease binding site in PRI must reside primarily or entirely in repeats 1, 2, 5, or 7, the short N- or C-terminal segments, or a combination of these. Deletion of repeat units 3–5, 5–6, or 5 alone results in mutants that exhibit only binding activity. Hence, the binding site cannot reside exclusively in repeat 5. Other internal deletions or N- or C-terminal deletions of 6–86% of the protein all abolish activity. These results suggest that PRI has a modular structure, with one primary structural repeat constituting one module. The approach taken may be applicable to other proteins with repeat structures.

Duplication of protein sequences is a strategy commonly used by nature to generate macromolecular structures from what may be a finite number of protein structural motifs (1). The uses of duplication are diverse and include, for example, the generation of large structural spacers, ligand-binding clefts, and multiple active sites. Of fundamental interest in the study of proteins arising from sequence duplication is the assessment of contributions of individual repeats to the function of the molecule as a whole. An approach toward analyzing repeats in one protein may therefore have applicability to other proteins with repeat structures.

Human placental ribonuclease inhibitor (PRI) is a 50-kDa protein that consists predominantly of seven tandem leucine-rich repeats, each exactly 57 residues long and ≈40% identical to one another (2–4). PRI is a member of a family of cytoplasmic proteins present in a wide variety of mammalian tissues that form extremely tight complexes with both secretory and intracellular ribonucleases (5). These proteins may play a role in controlling cytoplasmic RNA levels (5).

Although PRI has seven internal repeats, inhibition occurs with only a 1:1 stoichiometry (6). PRI competitively inhibits bovine pancreatic ribonuclease A (RNase A) with an extremely low K_i value of 4×10^{-14} M (7, 8), approaching the affinity of avidin for biotin (9). PRI binds with an even lower K_i value of 7×10^{-16} M to angiogenin, a blood vessel-inducing protein with 35% sequence identity to pancreatic RNase, thereby abolishing both its ribonucleolytic and angiogenic activities (7, 8, 10–12).

In this regard, mapping of the angiogenin/RNase binding site in PRI may serve both as a basis for the rational design

of antiangiogenesis agents as well as a foundation for understanding the unusually high affinity of this interaction. Because PRI is a large protein and there is currently no information on where the binding site may be, site-specific mutagenesis could be excessively time-consuming. As an alternative, we exploited the internal-repeat structure of PRI by deleting these repeats either singly or in combination and then testing the resulting mutants for activity.

Two salient features of our approach were as follows. (i) Precise integral multiples of 57 residues were deleted because that is the exact length of a repeat unit in PRI. (ii) Deletion boundaries were based on a set of repeat unit boundaries that begins at residue 30 and ends at residue 428 because the strong repeat motif of PRI begins and ends at approximately the same residues (2). These boundaries would therefore seem reasonable choices for defining potential structural units in PRI. The systematic internal-deletion mutagenesis approach that results, which we call “modular” mutagenesis, allows us to show that certain selective internal-repeat deletions, but not N- or C-terminal deletions, are compatible with activity.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized on a Biotix model 102 DNA synthesizer. RNase A–Sephacrose was prepared by the method of Blackburn (13). The sources of all other materials have been described (2, 7, 8, 14).

Recombinant Plasmid Construction. All recombinant plasmids were derived from pTRP-PRI, which contains the *Escherichia coli trp* promoter and the entire PRI coding sequence (15). Details of the constructions, which involved the use of restriction enzymes, synthetic oligonucleotide duplexes, and DNA ligase, are not given here due to space limitations but will be made available upon request. Aside from the portions of PRI either deleted or substituted for, the native PRI amino acid sequence has been preserved. Exceptions are as follows, with the residue numbers denoting their positions in the native protein (2): PRIΔ4–7, Gln-430 → Ala and Val-432 → Lys; PRIΔ1–7, Glu-429 → Gln, Gln-430 → Ala, and Val-432 → Lys; PRIΔ1–3, Glu-201 → Gln; PRIΔNα, Arg-63 → Gly; PRIΔCα, Tyr-434 → Ser; PRIΔCδ, Arg-63 → Pro.

The identities of all recombinant plasmids were confirmed by a combination of restriction enzyme mapping and sequencing of all regions in which synthetic oligonucleotide duplexes had been incorporated (16, 17).

Bacterial Extract Preparation. Forty milliliter cultures of *E. coli* strain W3110 transformed with the recombinant plasmids were grown and induced as described (15). Four to 5 hr after

induction, 36 ml of each culture was centrifuged at $3600 \times g$ for 10 min at 4°C . The pellets were resuspended in 5 ml of 50 mM Hepes, pH 7/100 mM NaCl/1 mM EDTA/15% glycerol (vol/vol) (buffer A) supplemented with 10 mM dithiothreitol and 1 mM phenylmethanesulfonyl fluoride and sonicated at 4°C in two 10-sec cycles with a Branson model 350 sonifier at power setting 7 on pulse (70% on per sec). The sonicates were centrifuged at $15,600 \times g$ for 15 min at 4°C to remove insoluble debris, yielding a soluble extract.

Assays. *p*-Hydroxymercuribenzoate (pHMB)-sensitive binding to RNase A-Sepharose. All procedures were done at 4°C . The extract, 1.4 ml, was added to 50 μl of RNase A-Sepharose in a 1.5-ml centrifuge tube, and the mixture was rotated end-over-end at 20 rpm for 30 min. The mixture was centrifuged at $6000 \times g$ for 1 min, and the supernatant was discarded. The resin was washed five times. The first three washes consisted of adding 1 ml of buffer A supplemented with 5 mM dithiothreitol, end-over-end rotation for 15 min, and centrifugation as above. The last two washes consisted of adding 1 ml of buffer A, end-over-end rotation for 5 min, and centrifugation as above. Elution was achieved by the addition of 50 μl of 0.1 M Tris, pH 8/0.1 M NaCl/2 mM pHMB, mixing by gentle pipetting, and incubation for 30 min followed by centrifugation as above. Twenty microliters of the eluate were added to sample buffer, subjected to NaDodSO₄/PAGE, transferred to nitrocellulose, and immunoblotted as described (14, 18, 19).

Inhibition of RNase A. The extract was assayed for inhibition of RNase A activity toward the dinucleotide cytidyl(3'-5')adenosine under described conditions (7). The RNase A and substrate concentrations were 0.15 nM and 100 μM , respectively. To control for small variations in the concentration of cellular constituents in the extract, the volumes of extract assayed were normalized to the A_{260} of the extract.

RESULTS

The approach taken to map the angiogenin/RNase binding site in PRI involved mutagenesis of the PRI cDNA followed by expression of the mutant proteins in *E. coli*. Two assays were used for screening *E. coli* extracts containing PRI mutants for activity. One assay involved testing the mutants in these extracts for pHMB-sensitive binding to RNase A-Sepharose; the RNase A-native PRI complex is completely dissociated by treatment with pHMB (6). The other assay involved testing the extracts containing these mutants for inhibition of RNase A enzymatic activity. In this assay, comparison was made to a negative control extract, in which PRI is absent, that displays a background inhibition toward RNase A.

In the first series of 12 mutants, from one to all seven internal-repeat units were deleted in various combinations (Fig. 1A). Two mutants, in which repeats 3-4 or repeat 6 were deleted (PRI Δ 3-4 and PRI Δ 6, respectively), both bind to and inhibit RNase A. Thus, immunoblots of pHMB eluates of RNase A-Sepharose that had been incubated with the mutant-containing extracts display immunoreactive bands of about the expected sizes, ≈ 38 and 44 kDa for PRI Δ 3-4 and PRI Δ 6, respectively (Fig. 2A, lanes 20 and 24), indicating that these mutants bind to RNase A. Furthermore, inhibition of RNase A activity occurs at extract concentrations significantly lower than that of the negative control extract in which PRI is absent (Fig. 3), strongly suggesting that these mutants also inhibit RNase activity.

Three additional mutants, PRI Δ 3-5, PRI Δ 5, and PRI Δ 5-6, bind to RNase A, as evidenced by the immunoreactive bands of about the expected sizes of ≈ 31 , 44, and 38 kDa, respectively, in their pHMB eluates (Fig. 2A, lanes 21-23). However, the intensities of these bands are significantly less than

FIG. 1. RNase A binding and inhibitory activities of PRI mutants. (A) Internal-deletion mutants. In the diagram of native PRI, numbers refer to repeats defined as follows: 1, residues 30-86; 2, 87-143; 3, 144-200; 4, 201-257; 5, 258-314; 6, 315-371; 7, 372-428. In the nomenclature of internal-deletion mutants, numbers refer to deleted repeats. (B) N- and C-terminal deletion mutants. Residues deleted are 1-62 (Δ N α), 1-225 (Δ N β), 1-298 (Δ N γ), 1-394 (Δ N δ), 435-460 (Δ C α), 396-460 (Δ C β), 227-460 (Δ C γ), and 64-460 (Δ C δ). (C) Additional PRI mutants. In the diagram of native PRI, letters refer to repeats defined as follows: A, residues 54-110; B, 111-167; C, 168-224; D, 225-281; E, 282-338; F, 339-395. In PRI(ABC)₂ and PRI(DEF)₂, repeats A-C substitute for repeats D-F, and vice versa, respectively. In the diagrams of PRI-CP1 and PRI-CP2, numbers designate residue positions in the native protein, and letters indicate a tetrapeptide inserted between original residues 460 and 1 in PRI-CP2.

those for native PRI or mutants PRI Δ 3-4 and PRI Δ 6, and none of these mutants inhibits RNase A under the assay conditions used. [For simplicity, we will refer to full-length PRI both expressed in *E. coli* and isolated from human placenta as native PRI. Recombinant PRI has physicochemical, immunological, and inhibitory characteristics virtually identical to those of PRI isolated from placenta, save for the

FIG. 2. Assay of the PRI mutants for pHMB-sensitive binding to RNase A-Sepharose. Twenty microliters of *E. coli* extract or pHMB eluate from RNase A-Sepharose obtained as described were added to sample buffer, subjected to NaDodSO₄/PAGE, and transferred to nitrocellulose. The filters were probed with affinity-purified, polyclonal, anti-PRI antibodies prepared as described (2). Molecular mass standards (Pharmacia LKB Biotechnology), in kDa, are indicated at right. (A) Immunoblot of extracts (lanes 1–14) or eluates (lanes 15–28) for assay of native PRI (lanes 1 and 15) or internal-deletion mutants PRI Δ 1 (lanes 2 and 16), PRI Δ 1-3 (lanes 3 and 17), PRI Δ 2 (lanes 4 and 18), PRI Δ 2-4 (lanes 5 and 19), PRI Δ 3-4 (lanes 6 and 20), PRI Δ 3-5 (lanes 7 and 21), PRI Δ 5 (lanes 8 and 22), PRI Δ 5-6 (lanes 9 and 23), PRI Δ 6 (lanes 10 and 24), PRI Δ 4-7 (lanes 11 and 25), PRI Δ 7 (lanes 12 and 26), and PRI Δ 1-7 (lanes 13 and 27). Also shown are data (lanes 14 and 28) for negative control extract (Δ PRI) prepared from *E. coli* transformed with pTRP- Δ PRI, a plasmid in which the entire PRI coding sequence has been deleted. (B) Immunoblot of extracts (lanes 1–10) or eluates (lanes 11–20) for assay of native PRI (lanes 1 and 11), N-terminal deletion mutants PRI Δ N α (lanes 2 and 12), PRI Δ N β (lanes 3 and 13), PRI Δ N γ (lanes 4 and 14), PRI Δ N δ (lanes 5 and 15), or C-terminal deletion mutants PRI Δ C α (lanes 6 and 16), PRI Δ C β (lanes 7 and 17), PRI Δ C γ (lanes 8 and 18), and PRI Δ C δ (lanes 9 and 19). Also shown are data (lanes 10 and 20) for Δ PRI extract. (C) Immunoblot of extracts (lanes 1–6) or eluates (lanes 7–12) for assay of native PRI (lanes 1 and 7) or mutants PRI(ABC)₂ (lanes 2 and 8), PRI(DEF)₂ (lanes 3 and 9), PRI-CP1 (lanes 4 and 10), and PRI-CP2 (lanes 5 and 11). Also shown are data (lanes 6 and 12) for Δ PRI extract.

absence of a blocked N terminus (15).] Seven other internal-repeat deletion mutants, including other single- and multiple-repeat deletion mutants, neither bind to nor inhibit RNase A (Fig. 1A). For the six mutants excluding PRI Δ 1-7, the lack of activity cannot be attributed to the absence of the recombinant protein because immunoreactive bands of roughly comparable intensity and of the appropriate molecular mass are detected in the extracts (Fig. 2A).

In a second series of eight mutants, the N- or C-terminal segments of PRI were deleted along with variable portions of the internal-repeat structure (Fig. 1B). In contrast to the internal-deletion mutants, all N- and C-terminal deletion mutants neither bind to nor inhibit RNase A (Fig. 2B). Included among these mutants are ones in which only the N-terminal segment plus one-half of repeat 1 or only the C-terminal segment have been deleted (PRI Δ N α and PRI Δ C α , respectively). Mixed extracts containing complementary pairs of PRI fragments—i.e., PRI Δ N α and PRI Δ C δ , PRI Δ N β and PRI Δ C γ , or PRI Δ N δ and PRI Δ C β —were not inhibitory.

In a third series of mutants, the PRI primary structure was manipulated to test hypotheses regarding its tertiary structure (Fig. 1C). We have previously observed that each repeat of PRI is composed of homologous half-repeats (2); the boundaries between half-repeats thereby define six internal repeats, A–F (Fig. 1C). To test whether these repeats can substitute for one another, we created a pair of mutants, PRI(ABC)₂ and PRI(DEF)₂, in which repeats A, B, and C

substitute for repeats D, E, and F, and vice versa, respectively. Each mutant therefore possesses a direct repeat of a three-repeat unit segment.

A second pair of mutants, PRI-CP1 and PRI-CP2, is each circular permutations of the primary structure of PRI. In a circular permutation of an amino acid sequence, the sequence itself is preserved except at the N and C termini, which have been joined together. Both mutants here possess new N- and C-terminal residues at original residues 255 and 254, respectively. In one mutant, the original N- and C-terminal residues have simply been joined together, whereas in the other, the tetrapeptide sequence Gly-Pro-Asn-Ala, which is predicted to adopt a β -turn (20), has been inserted between these residues. All four mutants in this third series neither bind to nor inhibit RNase A (Fig. 2C).

DISCUSSION

In the present studies, we have used modular mutagenesis to detect nonessential portions of PRI and thereby determine which regions may contribute to its angiogenin/RNase binding site.

The most surprising result in these studies is that certain deletions of PRI are compatible with activity (Fig. 1); others, including all N- and C-terminal deletions, completely abolish it. Thus, mutants containing deletions of repeats 3 plus 4 (PRI Δ 3-4) or repeat 6 (PRI Δ 6) both inhibit RNase A, whereas all others do not (Fig. 3). These two deletions differ in both

FIG. 3. Assay of PRI mutants for inhibition of RNase A activity. Various volumes of extracts containing native PRI (Δ), PRI Δ 2 ($\blacktriangle$), PRI Δ 3-4 ($\bullet$), PRI Δ 5 ($\blacklozenge$), PRI Δ 6 ($\blacksquare$), or PRI Δ 7 ($\blacktriangledown$) were tested for inhibition of RNase A under described conditions. Also shown is an inhibition profile of a negative control extract prepared from *E. coli* transformed with pTRP- Δ PRI ($\circ$). Extracts containing all other PRI mutants, like those containing PRI Δ 2, PRI Δ 5, and PRI Δ 7, display inhibition profiles similar to that of the negative control extract. [The one exception is the extract containing PRI Δ 1, which reproducibly inhibits with an IC_{50} that is $\approx 60\%$ of that of the negative control extract. However, no pHMB-sensitive binding to RNase A-Sepharose is seen when this assay is performed either normally, at pH 7, or at pH 6 (50 mM Mes), the pH at which the inhibition assay is performed, suggesting that this shift in the inhibition profile is not due to the mutant protein. In addition, when sonication is performed at pH 6 (50 mM Mes) instead of the usual pH 7 and assayed either normally, at pH 6, or at pH 7 (0.1 M Hepes), no differences are observed between the experimental and control inhibition profiles. Under these conditions, extracts containing native PRI still inhibit at concentrations easily distinguishable from that of the negative control extracts, as in this figure.]

size and position in the primary structure of the protein. Extension of these deletions to include adjacent repeats abolishes inhibitory activity, as does deletion of other protein regions of comparable size.

These results collectively allow a mapping of the angiogenin/RNase binding site in PRI. Although how tightly PRI Δ 3-4 or PRI Δ 6 bind to angiogenin or RNase A is yet undetermined, the fact that inhibitory activity is seen at an RNase A concentration of 0.15 nM (Fig. 3) suggests that the K_i values for both mutants are subnanomolar. This, in turn, suggests that the main contact area in PRI is intact in both mutants and cannot be located in the deleted repeats—i.e., 3, 4, or 6 (Fig. 4). Therefore, the angiogenin/RNase contact region on PRI must be located predominantly or entirely in repeats 1, 2, 5, or 7, the N- or C-terminal segments, or a combination of these. Further, the contact region cannot reside exclusively in repeat 5 because three mutants in which this repeat has been deleted, PRI Δ 3-5, PRI Δ 5, and PRI Δ 5-6, all bind to RNase A (Fig. 2A).

These three mutants, however, do not inhibit RNase A (Fig. 1A), and two plausible explanations, which are not mutually exclusive, are as follows. (i) Binding to RNase A is sufficiently weakened that activity is observed only in the more sensitive of the two assays, the binding assay, where

FIG. 4. Mapping of angiogenin/RNase binding site in PRI. Numbers refer to repeat units, N and C designate the N and C termini, and shaded background indicates nonessential repeats. The angiogenin/RNase binding site resides predominantly, or entirely, in repeats 1, 2, 5, 7, the N- or C-terminal segments, or a combination of these. Of these, the binding site cannot reside exclusively in repeat 5 (hatched).

the effective RNase A concentration is relatively high, $\approx \mu\text{M}$, based on a capacity for native PRI of ≈ 0.7 mg/ml resin reported for RNase A-Sepharose (13); this result compares with an RNase A concentration of 0.15 nM in the inhibition assay. (We therefore estimate that a PRI mutant must bind to RNase A with K_i values lower than $\approx \text{nM}$ or $\approx \mu\text{M}$ for activity to be detected in the inhibition or binding assays, respectively.) This would imply that, with high concentrations of mutant proteins, inhibition may be seen. (ii) Binding to RNase A occurs at a site outside of its active site and does not result in inhibition.

The present results bear upon the structural significance of the PRI repeat units. PRI Δ 3-4 is active, suggesting that repeat 2 can functionally substitute for repeat 4 because it may occupy a similar position in the mutant protein with respect to repeat 5 as repeat 4 does in the native protein. Along the same lines, repeat 5 appears capable of functionally substituting for repeat 3, and, applying this reasoning to the active mutant PRI Δ 6, repeats 5 and 7 each appear capable of functionally substituting for repeat 6. This analysis can also be extended to a lesser extent to the three additional mutants that display binding activity, PRI Δ 3-5, PRI Δ 5, and PRI Δ 5-6, and the results collectively suggest a significant degree of flexibility with respect to repeat substitutions that can be tolerated in the region of repeats 3-6.

This flexibility in allowable functional substitutions, in turn, suggests that PRI has a modular structure, with one 57-residue repeat constituting one structural module. The boundaries defining repeats 1-7 may define or nearly define these modules because selective deletions based on these boundaries result in functional proteins. In this regard, it will be of interest to determine whether deletions of nonintegral multiples of 57 residues or regions based on other repeat boundaries are compatible with activity.

The leucine-rich repeats of other proteins, which are close to, or exactly, 24 residues long and which are similar to the homologous half-repeats that comprise the 57-residue PRI repeat (2-4), may also constitute structural modules in their respective proteins. These proteins are of diverse function and include the platelet receptor for von Willebrand factor (21, 22), leucine-rich α_2 -glycoprotein (23), fibroblast proteoglycan core protein (24), yeast adenylate cyclase (25), and the *Drosophila* chaoptin and Toll proteins—the last two being factors involved in embryonic and photoreceptor cell development, respectively (26, 27). These repeats may mediate protein-protein or protein-membrane interactions (23, 26, 27). Modular mutagenesis of these proteins may, as with PRI, be of use in understanding their function.

The mutant PRI(ABC) $_2$, in which repeats A, B, and C substitute for repeats D, E, and F, is inactive, as is its complementary mutant, PRI(DEF) $_2$ (Fig. 1C). The absence of activity may be due to loss of critical contact regions in both mutants. For example, one-half of repeat 7 or one-half of repeat 1 plus repeat 2 have been substituted for in PRI(ABC) $_2$ and PRI(DEF) $_2$, respectively. Deletion of any of these three repeats individually abolishes activity (Fig. 1A).

Alternatively, the boundaries defining repeats A-F may not define the structural modules of PRI. A possible consequence is that the rearrangements may have interfered with the proper folding of the protein, a consideration that may also apply to the inactive deletion mutants. Indeed, mutagenesis of even single residues in other proteins can cause substantial destabilization of structure (28-30). Hence, the absence of activity in a PRI mutant does not necessarily imply that the regions either deleted or substituted for contribute directly to the contact site in PRI.

One potential implication of the flexibility in allowable functional substitutions in the region of repeats 3-6 is that these and perhaps all repeat units may be set in a regular or nearly regular array in three-dimensional space. In the ex-

treme case of a perfectly regular arrangement, the repeat units must form either a circle, a helix, or a line. Of these, a circular arrangement may bring the N and C termini into close proximity. This was tested experimentally by determining the activity of PRI mutants that are circular permutations of the primary structure; circular permutations of proteins with spatially proximal N and C termini in certain cases allow the attainment of active, native-like structures (31, 32).

The new N- and C-terminal residues in the permutations examined (Fig. 1C) were chosen to minimize potential detrimental effects of the new charges from the new termini; these residues occupy positions in the native primary structure that are predicted to be in a surface β -turn in the nonessential repeat 4 (2). The original N and C termini were brought together in different ways in the two permutations to maximize the possibility of allowing the attainment of a native-like structure. Neither mutant, however, is active (Fig. 1C), and from these results one cannot conclude whether or not the repeat units form a circle. Therefore, whether the three-dimensional arrangement of repeats in PRI, either entirely or in part, approximates a regular structure remains to be seen.

The internal-repeat structure of PRI probably arose by gene duplication. The present results now suggest that this duplication may have proceeded through functionally active intermediates, perhaps similar to some of the active mutants described, that possess less than a full complement of repeat units. Such a mechanism may have been operative in the evolution of proteins with repeat structures more generally.

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