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EXPRESSION OF HUMAN PLACENTAL RIBONUCLEASE INHIBITOR IN ESCHERICHIA COLI

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Summary: Human placental ribonuclease inhibitor (PRI) has been expressed in and isolated from Escherichia coli. Its apparent molecular weight, immunoreactivity and amino acid composition are virtually identical with those of native PRI. It inhibits the enzymatic activities of either angiogenin, a blood vessel inducing protein homologous to pancreatic RNase (RNase A), or RNase A in a stoichiometry of 1:1. Recombinant PRI binds to angiogenin and RNase A with K_i values of 2.9×10^{-16} M and 6.8×10^{-14} M, respectively, comparable to the affinities of native PRI for these enzymes. Thus, these results confirm that PRI inhibits angiogenin more effectively than RNase A.

Human placental RNase inhibitor (PRI) is one of a series of interrelated RNase inhibitors of M_r ~50 kDa in cytoplasmic extracts of many mammalian tissues (1,2). Its potency as an RNase inhibitor has led to its widespread use in in vitro transcription and translation reactions when preservation of RNA integrity is essential (3-5). PRI inhibits angiogenin (6) and RNase A with K_i values of 7.1×10^{-16} M and 4.4×10^{-14} M, respectively (7,8). PRI abolishes both the enzymatic and angiogenic activities of angiogenin (9). We have shown that the PRI binding site of angiogenin involves catalytic residues (10) and have isolated the PRI cDNA and determined its primary structure (11). The expression of recombinant human PRI, here described, will facilitate the identification of regions of PRI involved in binding angiogenin and, hence, the design of novel angiogenin inhibitors.

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Abbreviations: PRI, placental ribonuclease inhibitor; RNase, pancreatic ribonuclease; PMSF, phenylmethanesulfonylfluoride.

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MATERIALS AND METHODS

Materials. The plasmid pAng1 was a gift of Dr. R. Shapiro. Oligodeoxynucleotides were from Biotix. Angiogenin for PRI kinetic analysis or enzymatic assay was obtained from eukaryotic or prokaryotic expression systems, respectively (12,13). Sources of all other materials have been described (7,8,10,11,13).

Construction of PRI Expression Plasmid. The plasmid pAng1 (13), which contains the E. coli trp promoter, was digested with Kpn I, treated with T4 DNA polymerase to create blunt ends, and then digested with BstE II. The large fragment was ligated with a synthetic oligodeoxynucleotide duplex containing the portion of the E. coli trpL translation initiation sequence (14) that is missing in this large fragment and an initiator ATG codon (Figure 1). The product was digested with BstX I and EcoR I, and the large fragment was ligated with the 1.6 kb fragment obtained by identical digestion of pUC18-λPRI-A (11) to give pTRP-PRI. The 1.6 kb fragment contains the entire PRI cDNA coding sequence save for the initiator codon. Sequencing (15) of the trpL translation initiation sequence of pTRP-PRI confirmed its authenticity.

Expression and Isolation of PRI. Four liters of M9 medium supplemented with 50 μg/ml ampicillin, 0.4% glucose, and 0.4% casamino acids were inoculated with 40 ml of an overnight culture of E. coli strain W3110 transformed with pTRP-PRI and shaken at 37°C. At an OD_{600} = 1.0 (~3 hr), 3-β-indoleacrylic acid and glucose were added to final concentrations of 20 μg/ml and 0.4%, respectively. After an additional 4-6 hr, the culture was centrifuged at 4,000 g for 10 min at 4°. All subsequent steps were performed at 0-4°C. The bacterial pellet (~20 g), either fresh or frozen overnight at -20°C, was resuspended in 32 ml of 10 mM potassium phosphate, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 10 mM DTT, and 1 mM PMSF and sonicated in 6 ten-s cycles with a Branson Model 350 Sonifier at power setting 7 on pulse (70% on per s). The sonicate was centrifuged at 100,000 g for 1 hr. The supernatant was made 1% in streptomycin sulfate, and after 30 min, the suspension was centrifuged at 17,300 g for 30 min. The supernatant was diluted with four volumes of 50 mM potassium phosphate, pH 6.4, containing 1 mM EDTA, 5 mM DTT, and 15% glycerol (vol/vol) (buffer A) and applied at a flow rate of 0.5 ml/min to a column containing 2 ml of RNase A-Sepharose and equilibrated with buffer A (16). Washing of the column, necessary for removal of a substantial amount of nonspecifically bound material, and elution were performed as described (16).

Protein Assays. PRI activity was assayed by inhibition of RNase A activity (16). The ribonucleolytic activity of angiogenin and the concentrations of angiogenin and RNase A stock solutions were measured as described (7,17). In order to determine inhibition stoichiometries, PRI was first dialyzed in less than 2 hr against degassed water on a Centricon 30 microconcentrator (Amicon) at 4°C, immediately assayed for

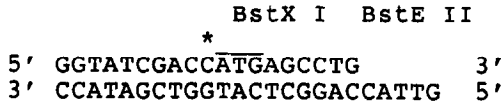

Figure 1: Sequence of synthetic oligonucleotide duplex employed for construction of pTRP-PRI. Restriction enzyme sites within, BstX I, or compatible with the ends, BstE II, of the oligodeoxynucleotide duplex are indicated. Between the blunt end and BstX I site is a portion of the trpL translation initiation sequence (14) save for the C indicated by the asterisk which substitutes for an A. Initiator ATG codon is indicated by the line.

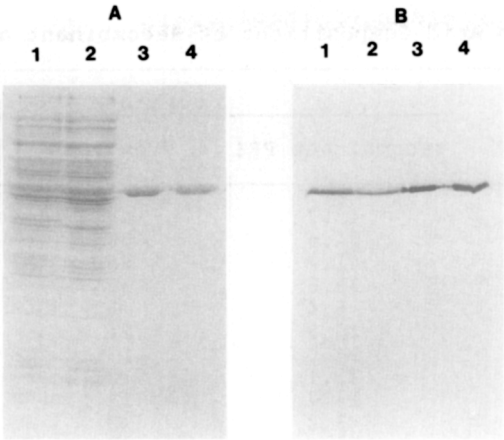

Figure 2: $NaDodSO_4$-PAGE (A) and immunoblot (B) analysis of samples from different steps of purification of recombinant PRI. Samples are crude E. coli lysate (lane 1), supernatant following streptomycin sulfate precipitation (lane 2), affinity column eluate (lane 3), and native PRI standard (lane 4). Amounts of protein loaded (lanes 1-4) are, in μg, (A) 40, 40, 2, 2 and (B) 60, 60, 0.5, 0.5. $NaDodSO_4$-PAGE was conducted using 12.5% separating gel with 5% stacking gel. Gel in (A) was stained with Coomassie Blue.

inhibition of angiogenin or RNase A, and then subjected to amino acid analysis to measure concentration. For specific activity measurements, PRI concentration was estimated from the A_{280} using a molar absorptivity based on its amino acid composition (18).

Physicochemical, Immunological, and Kinetic Analyses. $NaDodSO_4$-PAGE, immunoblotting, amino acid analysis, and automated Edman degradation were performed using established procedures (12,19,20,21). The association and dissociation rate constants for the interaction of recombinant PRI with angiogenin or RNase A were determined as described (7,8,10).

RESULTS AND DISCUSSION

PRI is a cytoplasmic protein which does not appear to require posttranslational modification for its activity (2), an ideal situation for direct expression in a prokaryote. The purification procedure described allows for the isolation of ~1 mg of PRI in greater than 98% purity from four liters of E. coli culture (Figure 2A). The specific activity of the recombinant protein, 110,000 units/mg, is essentially the same as that of native PRI (1,16).

Physicochemical and Immunological Characterization. The apparent molecular weight and immunoreactivity of recombinant PRI, as assessed by $NaDodSO_4$-PAGE and immunoblotting with anti-PRI antibodies, respectively, are indistinguishable from those of native PRI (Figure 2). The amino acid composition of recombinant PRI agrees well with that predicted by the cDNA and that of native PRI (Table 1). Edman degradation of 0.5 nmol of

Table 1. Amino Acid Compositions of Recombinant and Native PRI[a]

amino acid	residues/mol		
	recombinant PRI	from cDNA[b]	native PRI[c]
Asx	43.7	44	47
Glx	54.6	59	64
Ser	41.7	45	45
Gly	33.8	32	36
His	5.4	5	6
Arg	24.8	24	23
Thr	14.0	13	16
Ala	33.1	32	34
Pro	14.7	14	17
Tyr	3.2	3	4
Val	23.7	25	24
Met	1.3	2	2
Ile	11.2	12	12
Leu	93.5	92	85
Phe	4.3	4	6
Lys	19.2	16	17
Cys	30.4	32	30
Trp	5.5	6	5

[a] By Pico-Tag method (20). Cys determined as cysteic acid after performic acid oxidation. Trp determined after hydrolysis with methanesulfonic acid. Average of analyses for three separate preparations. [b] Excluding initiator Met. From Lee et al. (11). [c] From Blackburn and Moore (2).

recombinant PRI resulted in the sequence Ser-Leu-Asp-Ile-Gln-Ser-Leu-Asp which corresponds exactly to that of residues 1 to 8 deduced from the cDNA (11). This shows that the initiator Met has been removed from the recombinant protein. Native PRI is blocked at the α-amino group of the residue found at the N-terminus of recombinant PRI (11).

Kinetic Characterization. Recombinant PRI inhibits the enzymatic activity of either angiogenin or RNase A in a 1:1 stoichiometry (not shown). Treatment with the sulfhydryl reagent, p-(hydroxymercuri)benzoate (1 mM in the assay), completely reverses PRI inhibition of RNase A. These results are identical with those obtained for native PRI (1,9).

Tryptophan fluorescence is enhanced 50% upon formation both of the angiogenin·recombinant PRI (not shown) and the angiogenin·native PRI complex (7) and served to monitor the association of the two proteins. The dependence of the pseudo-first-order rate constant of association on angiogenin concentration is consistent with the two-step mechanism described for angiogenin and native PRI (7) in which the first step, a rapid equilibrium resulting in the formation of an enzyme·inhibitor complex, is followed by the second, a slower isomerization to a tighter complex. The values for K_1, the

Table 2. Inhibition of Angiogenin and RNase A by Recombinant and Native PRI[a]

enzyme	inhibitor	K_1 (μM)	k_2 (s^{-1})	$k_2/K_1 \times 10^{-8}$ ($M^{-1}s^{-1}$)	$k_{-2} \times 10^{7}$ (s^{-1})	K_i (fM)
angiogenin	rPRI[b]	0.67	120	1.8	0.52	0.29
angiogenin	nPRI[b]	0.53	97	1.8	1.3	0.71
RNase A	rPRI[b]	ND[c]	ND	2.5	170	68
RNase A	nPRI[b]	ND	ND	3.4	150	44

[a]Conditions are 0.1 M Mes, pH 6, 0.1 M NaCl, 1 mM EDTA, and 25°C. Recombinant and native PRI are denoted by rPRI and nPRI, respectively. K_1, k_2, and k_{-2} refer to the two-step mechanism

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

where E is enzyme, I is inhibitor, and * denotes a tighter form of the EI complex (7). [b]Values are from Lee et al. (7,8). [c]ND, not determined.

dissociation constant of the first step, and k_2, the rate constant of the second step, are comparable to those for angiogenin and native PRI (Table 2), as are the apparent second-order rate constants of association ($\approx k_2/K_1$) of angiogenin or RNase A with PRI, both of which are close to the diffusion-controlled limit.

The dissociation rate constants of the angiogenin·recombinant PRI and RNase A·recombinant PRI complexes were determined by monitoring the release of free enzyme from the complex as a function of time (8). As with native PRI, recombinant PRI dissociates from its complex with angiogenin with a rate constant, k_{-2}, that is over 100-fold slower than that for its complex with RNase A (Table 2). The K_i values for angiogenin and RNase A inhibition, calculated from the association and dissociation rate constants, are comparable to those of native PRI and are both extremely low (Table 2). Recombinant PRI binds to angiogenin with a K_i value of 0.29 fM, which is actually ~2-fold lower than that for native PRI; it binds to RNase A with a K_i value of 68 fM. Thus, these results confirm that PRI inhibits angiogenin more effectively than RNase A.

The data demonstrate that E. coli can express a PRI whose physicochemical, immunological, and kinetic characteristics are virtually identical to those of native PRI, save for the absence of a blocked N-terminus. This equivalence confirms that eukaryotic posttranslational modifications are not essential for PRI activity. The purification procedure described evades the

hazards associated with isolation of PRI from human tissues and allows for the isolation of 1 mg of recombinant PRI in two days. Such a system will be of great advantage in determining the regions of PRI which form the binding site for angiogenin and RNases and may ultimately aid in the design of novel antiangiogenesis agents.

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REFERENCES

1. Blackburn, P., Wilson, G., and Moore, S. (1977) J. Biol. Chem. 252, 5904-5910.
2. Blackburn, P., and Moore, S. (1982) Enzymes (3rd. Ed.) 15, 317-433.
3. Robbi, M., and Lazarow, P. B. (1978) Proc. Natl. Acad. Sci. USA 75, 4344-4348.
4. Scheele, G., and Blackburn, P. (1979) Proc. Natl. Acad. Sci. USA 76, 4898-4902.
5. de Martynoff, G., Pays, E., and Vassart, G. (1980) Biochem. Biophys. Res. Commun. 93, 645-653.
6. Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., and Vallee, B. L. (1985) Biochemistry 24, 5480-5486.
7. Lee, F. S., Auld, D. S., and Vallee, B. L. (1989) Biochemistry 28, 219-224.
8. Lee, F. S., Shapiro, R., and Vallee, B. L. (1989) Biochemistry 28, 225-230.
9. Shapiro, R., and Vallee, B. L. (1987) Proc. Natl. Acad. Sci. USA 84, 2238-2241.
10. Lee, F. S., and Vallee, B. L. (1989) Biochemistry, in press.
11. Lee, F. S., Fox, E. A., Zhou, H.-M., Strydom, D. J., and Vallee, B. L. (1988) Biochemistry 27, 8545-8553.
12. Kurachi, K., Rybak, S. M., Fett, J. W., Shapiro, R., Strydom, D. J., Olson, K. A., Riordan, J. F., Davie, E. W., and Vallee, B. L. (1988) Biochemistry 27, 6557-6562.
13. Shapiro, R., Harper, J. W., Fox, E. A., Jansen, H.-W., Hein, F., and Uhlmann, E. (1988) Anal. Biochem. 175, 450-461.
14. Nichols, B. P., and Yanofsky, C. (1983) Methods Enzymol. 101, 155-164.
15. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
16. Blackburn, P. (1979) J. Biol. Chem. 254, 12484-12487.
17. Shapiro, R., Weremowicz, S., Riordan, J. F., and Vallee, B. L. (1987) Proc. Natl. Acad. Sci. USA 84, 8783-8787.
18. Wetlaufer, D. B. (1962) Adv. Prot. Chem. 17, 303-390.
19. Laemmli, U. K. (1970) Nature (London) 227, 680-685.
20. Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. (1984) J. Chromatogr. 336, 93-104.
21. Strydom, D. J., Harper, J. W., and Lobb, R. R. (1986) Biochemistry 25, 946-951.