Synthesis of New Peptides with Prolactin-releasing Activity by a Combination of Recombinant DNA Technology and a Cysteine-specific Cyanylation Reaction

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A newly isolated peptide from bovine hypothalamus with prolactin-releasing activity (prolactin-releasing peptide; PrRP) was synthesized by a combination of recombinant DNA technology and a cysteine-specific cyanylation reaction, together with rat and human homologs. The peptides were expressed in the form of fusion proteins with basic fibroblast growth factor mutein, which were purified by heparin-affinity chromatography. The fusion proteins were cleaved at the cysteine residues of the junction site by cyanylation, followed by treatment with ammonia for C-terminal amidation. Purification of the resulting crude peptides was performed using chromatography on a gel-filtration column, a cation-exchange column, and a reversed-phase column. As an example, about 90 mg of bovine PrRP (bPrRP) was obtained from 20 l of culture bloth. The purified bPrRP showed full biological activities in binding to its receptor expressed on CHO cells and releasing arachidonic acid metabolite from the same cells, while the C-terminal acid form of bPrRP had little of these activities. These results indicate that the C-terminal amide structure is very important for expressing biological activity. The peptides obtained here might be very useful for studies on their biological significance and roles *in vivo*.

Key wors prolactin-releasing peptide; S-cyanylation; fusion protein

Recently a new peptide with prolactin-releasing activity was isolated in our Research Division from bovine hypothalamic tissue extract utilizing an orphan seven-transmembrane receptor (hGR3).[1] The cDNA structures of this peptide, and the corresponding human and rat peptides, indicated that 31 amino acid residues with a C-terminal amide group are produced as mature peptides (Fig. 1) from preproproteins. The biologically active peptide was mainly expressed in the rat hypothalamus and it promoted prolactin secretion from rat anterior pituitary cells. Based on these results, the peptide might be a potent candidate for the prolactin-releasing factor, which has not yet been identified. Therefore this peptide was named prolactin-releasing peptide (PrRP).

For the preparation of biologically active peptides by recombinant DNA technology, a major concern is how to avoid endogenous proteolysis. Some techniques for overcoming this difficulty have been developed: 1. accumulation in the extracellular or periplasmic region;[2,3] 2. use of hosts which have low proteolytic activities;[4] and 3. expression as a fusion protein, which has advantages in the purification steps, followed by specific cleavage.[5,6]

Previously we reported a novel process in which CS23, a basic fibroblast growth factor mutein,[7] was used as a fusion partner.[8] Two major advantages of this technique are as follows. First, heparin-affinity chromatography made it easy to purify the fusion protein in high yield. Second, introduction of the cysteine residue at the junction gave a reliable site-specific cleavage after treatment with cyanylation reagents such as 1-cyano-4-(dimethylamino) pyridinium tetrafluoroborate (DMAP-CN). Moreover, this method can yield the C-terminal amide form by treatment of the cyanylated protein with ammonia,[9] while the C-terminal acid form can be obtained by treatment with alkali such as sodium hydroxide.

Some procedures for the preparation of the peptide amide from recombinant products have been reported,[10—12] but compared with these methods, we found our process to be simpler and more productive. In this paper we describe the synthesis of bovine, rat, and human PrRP (bPrRP, rPrRP, and hPrRP) using our new technique.

Figure 2 shows the strategies for preparation of PrRPs. We constructed a bPrRP-CS23 expression vector (pTB960-10) by replacing the insulinotropin structural gene of pTB960-7, described in a previous paper,[8] with the synthetic bPrRP gene.[13] The transformant *Escherichia coli* MM294 (DE3)[14] carrying pTB960-10 was cultivated, the cells were collected by centrifugation, and the cell-free extract was obtained by sonication. Then the resulting bPrRP-CS23 fusion protein was purified by heparin-affinity column chromatography.[15]

bovine PrRP	H-Ser	Arg	Ala	His	Gln	His	Ser	Met	Glu	Ile	Arg	Thr	Pro	Asp	Ile	Asn	Pro	Ala	Trp	Tyr
rat PrRP	H-Ser	Arg	Ala	His	Gln	His	Ser	Met	Glu	Thr	Arg	Thr	Pro	Asp	Ile	Asn	Pro	Ala	Trp	Tyr
human PrRP	H-Ser	Arg	Thr	His	Arg	His	Ser	Met	Glu	Ile	Arg	Thr	Pro	Asp	Ile	Asn	Pro	Ala	Trp	Tyr

Ala	Gly	Arg	Gly	Ile	Arg	Pro	Val	Gly	Arg	Phe	-NH_2
Thr	Gly	Arg	Gly	Ile	Arg	Pro	Val	Gly	Arg	Phe	-NH_2
Ala	Ser	Arg	Gly	Ile	Arg	Pro	Val	Gly	Arg	Phe	-NH_2

Fig. 1. Amino Acid Sequences of Bovine, Rat, and Human PrRPs

Amino acid sequences are written in three-letter codes.

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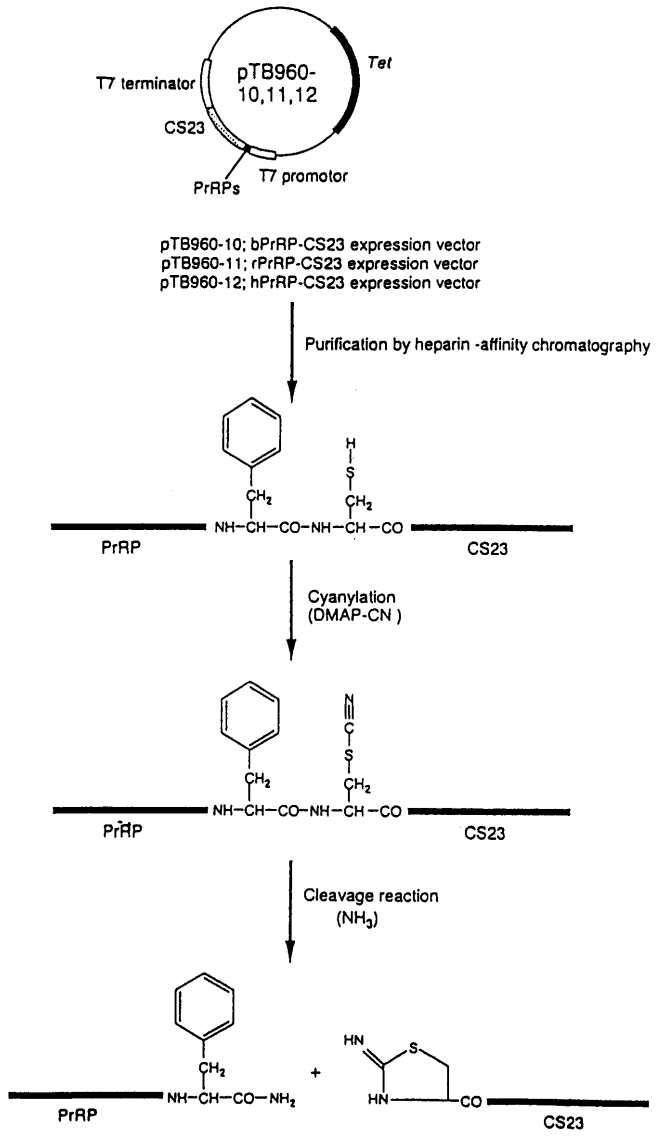

Fig. 2. Strategies for Preparation of PrRPs

The SH groups of the bPrRP-CS23 fusion protein were converted to SCN groups by DMAP-CN. The modified protein was cleaved by treatment with ammonia and then passed through a column of Sephadex G-25.[16] The resulting crude product was purified by chromatography on SP-5PW and C4P-50,[17] and approximately 90 mg of the purified bPrRP was obtained from 20 l of culture broth. To confirm the structural identity of the purified bPrRP, protein chemical analysis was performed. The results of N-terminal amino acid sequence analysis and amino acid composition analysis were in good agreement with those predicted from the cDNA sequence. The C-terminal residue of this peptide could not be detected by hydrazinolysis, indicating that the C-terminal residue is in the amide form. rPrRP and hPrRP were also obtained in essentially the same manner as described above. Moreover, the C-terminal acid form of bPrRP was obtained by alkaline hydrolysis after the cyanylation reaction. The gel filtration profile, SDS-PAGE pattern, and reversed-phase HPLC (Fig. 3) of the purified PrRPs showed high homogeneity. The purified bPrRP showed a retention time identical to that of a chemically synthesized standard and it gave the expected result upon molecular weight measurement by fast

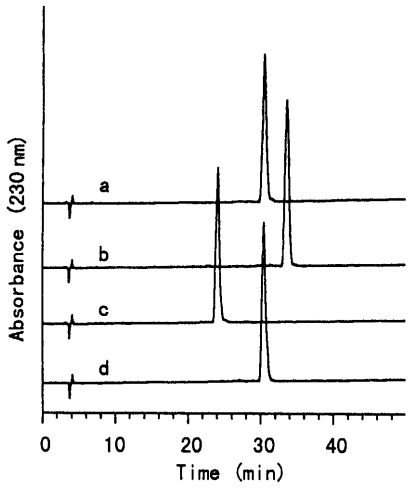

Fig. 3. Reversed-phase HPLC of the Purified PrRPs

(a) bovine PrRP (amide form); (b) bovine PrRP (acid form); (c) rat PrRP (amide form); (d) human PrRP (amide form) 5 μg of peptides was applied to an ODP-50 column (Shodex, 0.46×15 cm) and eluted with a linear gradient of acetonitrile (20—30%) in the presence of 0.1% trifluoroacetic acid.

atom bombardment mass spectrometry (MH^+, *m/z* observed: 3577.1 *vs.* theoretical: 3577.1).

The purified bPrRP (amide form) was subjected to biological assays with CHO cells which expressed the bPrRP receptor, showing that the bPrRP (amide form) and a chemically synthesized standard (amide form) equally inhibited the binding of [^{125}I]-labelled bPrRP. Also, this bPrRP (amide form) showed the same ability as an authentic sample in releasing arachidonic acid metabolite from the CHO cells. In contrast, the C-terminal acid form could not bind to the receptor, strongly suggesting that the C-terminal amide structure is essential for the binding of bPrRP to its receptor and the expression of biological activity.

Thus we obtained bPrRP, rPrRP, and hPrRP in large quantity and with a high degree of purity using a combination of recombinant DNA technology and a cysteine-specific chemical cleavage reaction. The peptides obtained here might be very helpful for clarification of their biological significance and roles *in vivo*. Studies along this line are now underway in our Research Division.

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13) Six synthetic oligonucleotides designed to have E. coli bias codons for the bPrRP structural gene and cleavage site were annealed and inserted into *XbaI-AvaI* cloning site of pTB960-7.
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15) The cell-free extract was applied to an AF-Heparin Toyopearl 650 M column (Tosoh, 3×50 cm) equilibrated with 50 mM phosphate buffer (pH 6.0). The column was washed and proteins were eluted with a linear gradient of NaCl (0—2 M).
16) 6 M urea was dissolved in the fusion protein solution followed by addition of 0.1 M acetic acid. After the addition of 2.4 mM of DMAP-CN, the reaction mixture was incubated at 25 °C for 15 min. This mixture was applied to a Sephadex G-25 column (Amersham Pharmacia Biotech, 4.6×60 cm) equilibrated with 50 mM phosphate buffer (pH 6.0). The main fraction was concentrated and 6 M urea and 3 M ammonia were added followed by incubation at 25 °C for 15 min. The mixture was gel filtrated with a Sephadex G-25 column as described above.
17) The reaction mixture was applied to a TSK Gel SP-5PW column (Tosoh, 2.15×15 cm) equilibrated with 50 mM phosphate buffer (pH 6.0) containing 3 M urea. The protein was eluted with a linear gradient of NaCl (0—0.35 M). The desired fraction was applied to a C4P-50 column (Showdex, 2.15×30 cm) and eluted with a linear gradient of 16—32% acetonitrile in the presence of 0.1% trifluoroacetic acid.

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