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AMINO ACIDS IN THE AMINO TERMINAL REGION OF THE RAT PROLACTIN CONTRIBUTE TO PRL-RECEPTOR BINDING AND NB2 CELL PROLIFERATION ACTIVITY

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To elucidate the structure-function relationship of rat prolactin, seven conserved or semi-conserved amino acids in the amino terminal region were investigated by site-directed mutagenesis. The antigenicity and electrophoretic mobility, as well as receptor binding and Nb2 cell proliferation in various mutants were assayed. Mutants with the cysteine at position 9 replaced with glycine (C9G), as well as R19G, S24A, Y26S, Y42F and Y42S, decreased the activities of receptor binding and Nb2 cell proliferation. A mutation of residue at 42 resulted in a loss of most of the activities. A mutation of residue at 39 (D39V and D39G) increased both receptor binding and cell proliferation activities. Thus, this study demonstrated that the seven amino acids in the amino terminal region contribute to the biological function of rat prolactin and that tyrosine at 42 is especially important.

Prolactins (PRLs) are one of the anterior pituitary hormones and they have several functions in a wide range of vertebrates. Despite several studies of the molecular basis, the versatility of PRL functions remains elusive. PRLs form a gene family including growth hormones (GH) and placental lactogens (PL) (1). Lactogenic activity is found in PLs and human growth hormone (hGH) as well as PRLs. Thus, the structure-function relationship of PRL is often argued in reference to the three-dimensional structure of GH, which has been analyzed by means of X-ray crystallography (2, 3). The binding of PRLs and hGH to the lactogenic receptors occurs through a molecular mechanism that depends upon a strict amino acid specificity (4, 5), suggesting that the apparent structural similarity to GH receives reevaluation.

We previously expressed recombinant rat (r) PRL in mammalian cells (6), that is biologically active PRL that is similar to that from the pituitary. The

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expression system does not require extraordinary manipulation, unlike the conventional production of recombinant PRL in the bacterial cells (4, 7). Using this expression system, we studied the structure-function relationship of the amino acid residues present in the evolutionarily variable amino terminal region of the rPRL, because much less is known about their roles. The results demonstrated that the amino acids in the amino terminal region are involved in receptor binding and Nb2 proliferation activities.

MATERIAL AND METHODS

Vector construction, mutagenesis and expression. Clones containing rPRL (rPRLSG) were constructed in an expression vector pSG5 (Stratagene, La Jolla, CA) by ligating rPRL cDNA (provided by Dr. R. A. Maurer, Iowa University) (8) cut with *Eco*RI, into the *Eco*RI site in the vector. All mutations in the amino terminal region of rPRL were generated by oligonucleotide-directed mutagenesis (9) using complementary oligonucleotides (Table 1) to replace the amino acids desired. All mutations were confirmed by sequence analysis.

Mutated clones were transfected with transformed COS-1 monkey kidney cells (supplied by Japanese Cancer Research Resources) using DEAE-dextran (10). PRLs were expressed in serum free GIT media (Wako Pure Chemicals, Osaka, Japan) and the cultured media was collected and stored for further analyses without purification as described (6).

Radioimmunoassay (RIA). RIA and competitive binding were performed by means of the double antibody method with a NIADDK RIA kit consisting of rPRL I-6 and anti-rPRL S-9, according to the procedure described in the instruction manual.

Electrophoresis and Western blotting. Electrophoreses on a 12.5% SDS-polyacrylamide gel (SDS-PAGE) and 12.5% non-denaturing polyacrylamide gel (ND-PAGE) were performed according to Laemmli (11) and Orstein and Davis (12, 13), respectively, followed by staining with Coomassie brilliant blue or electroblotting onto a nitrocellulose membrane. Western blotting was carried out by an incubation with rabbit anti-rPRL S-9, followed by a reaction with peroxidase-conjugated second goat anti-rabbit IgG antibody. The final color was developed using an H_2O_2 and horseradish peroxidase reagent (Konica, Tokyo, Japan).

Radio-receptor-binding test (RRA). RRA was performed as described by Sakai *et al.* (14). Rat PRL I-6 was labeled with ^{125}I using lactoperoxidase (15). Competitive binding in liver membrane of 20 days-pregnant rat (100 mg) was measured using ^{125}I-rPRL (about $1x10^5$ cpm) in the presence of recombinant rPRL followed by an overnight incubation at room temperature. The precipitates were collected by centrifugation at 11,000x*g* and the radioactivity was counted.

Nb2 cell proliferation. The proliferation activity of Nb2 node lymphoma cells was measured in Fischer's medium (GIBCO) containing 10% FCS as described (16). The medium was changed to the Fischer's medium containing 1% FCS and 10% horse serum (Flow Lab., McLearn, V.A.) 24 h before the assay. During the assay, the cells were dispersed in Fischer's medium containing 10% horse serum and placed in 96-well culture plats at $3\text{-}4.5x10^5$ cells/1.5 ml/well. Fifty microliters of medium containing various concentrations of recombinant rPRLs were added to the wells and incubated at 37°C in an atmosphere of 95% air-5% CO_2 for 72 h. Cell growth was measured colorimetrically using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (17).

RESULTS

PRLs contain conserved amino acids that mainly cluster in two regions of the central and carboxyl terminal regions (18, 19). On the other hand, the amino terminal region contains some conservative amino acids. To examine the functional roles of the amino terminal region, mutations were designed to replace some conservative and semi-conservative amino acids, as shown in Table 1. We obtained 13 mutated clones out of 16 (Fig. 1).

After transfecting COS-1 cells with mutant clones, culture media containing recombinant rPRL were analyzed as follows. The amounts of mutant PRL expressed in COS-1 cells measured by RIA varied (Fig. 2a), but the concentrations were sufficient for most of the following experiments. There were no significant changes in the antigenicity compared with that of rPRL I-6, except for the mutant (Y26S), which had a slight alteration in antigenicity (data not shown). The amount of Y26S was corrected by dye staining after gel electrophoresis. SDS-PAGE (Fig. 2b) and Western blotting (data not shown) showed that the molecular mass of recombinant PRL was the same as that of rPRL I-6. Five mutants varied in mobility on ND-PAGE (Fig. 2c) and in Western blots (Fig. 2d). These mutants contained a charge-difference (R19G, D39V and D39G), slight structural alterations by disruption of the disulfide bridge (C9G), or a deletion of phenolic ring (Y42S).

Figures 2e and 3 (upper panel) show the receptor binding activity of recombinant rPRLs. The activity of the mutations C9G, S24T and Y26F did not change. The activity decreased considerably (less than 63%) in the mutants R19G, S24A, Y26S, F48S, F48Y and F48L. A replacement of tyrosine (Y) at 42

Table 1. Design of oligonucleotide primers for mutation of rat prolactin

Residue No.	Amino acid change designed		Oligonucleotide designed
	from	to	
9	C	S/G	GTGGCGAC(A/G)GCCAGACACCT
19	R	A/G	TGTTTGACG(G/C)TGTGGTCATG
24	S	T/A	TCATGCTT(A/G)CTCACTAC
26	Y	F/S	TTCTCACT(T/C)CATCCATAC
39	D	V/G	TTGAATTTG(T/G)TAAACAGTA
42	Y	F/S	TAAACAGT(T/C)TGTCCAAG
48	F	S/L/P	ATCGTGAG(T/C)(T/C)TATTGCCA
48	F	Y	ATCGTGAGT(A)TATTGCCA

Nucleotides to be mutated are indicated in parentheses and the mixed nucleotides at a single point are indicated by a slash . Amino acids obtained as mutant clones are indicated by bold capitals.

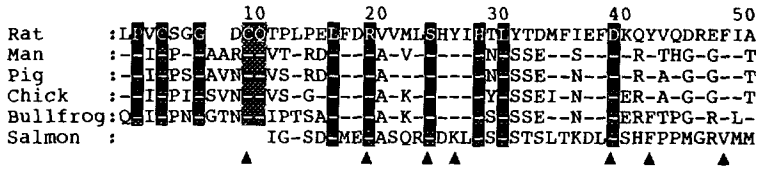

Fig. 1. Comparison of 50 amino acids in the amino terminal sequence of PRLs. Fifty amino acids in the amino terminal region of rPRL were compared with porcine (18), human (24), chick (25), bullfrog (26) and salmon (27) PRLs. Identical amino acids are indicated by (-) and the gaps (open space) were made to maximize the homology. Amino acids conserved throughout evolution are indicated by white letters. Amino acids to be replaced are indicated by closed triangle below .

with phenylalanine (F) or serine (S) dramatically decreased the receptor binding activity (less than 0.03%). On the other hand, the mutants D39V and D39G had about a 140 and 250% increase in the activity, respectively.

The Nb2 cell proliferation activity is shown in Figs. 2f and 3 (lower panel). The mutants S24T and Y26F, whose receptor binding activities did not change, also retained proliferation activity, but C9G decreased to about 32%. The mutants R19G, S24A, Y26S, F48S, F48Y and F48L, in which the receptor binding activities were decreased, showed comparatively low proliferation activity (3.5~71%), though only mutant F48L (30% binding activity) still had 88% activity. Two mutantions at residue 42, which has poor receptor binding activity, also caused a loss of most of the proliferation activity (less than 1%). The mutations D39V and D39G, which had increased receptor binding activities had high proliferation activity (175 and 217%, respectively).

Except for residues 9 (C) and 19 (R), two or three mutant clones were obtained for each residue. The results of the receptor binding and proliferation activities showed that the side chain structure of each amino acid influences the biological activities. The hydroxyl group at residue 24 (S), the aromatic ring at 26 (Y) and the phenolic group at 42 (Y) were also important. Cooperative changes in the receptor binding and the proliferation activities were not evident in C9G, R19G, S24A, Y26S and F48L. Replacing the negative charge at 39 enhanced the receptor binding and the proliferation activities.

DISCUSSION

The conserved amino acids in PRL are present largely in the central and carboxyl regions (18). Because sequence homology with GHs and PLs and the lactogenic activity common in primate GHs and PLs have been argued, the

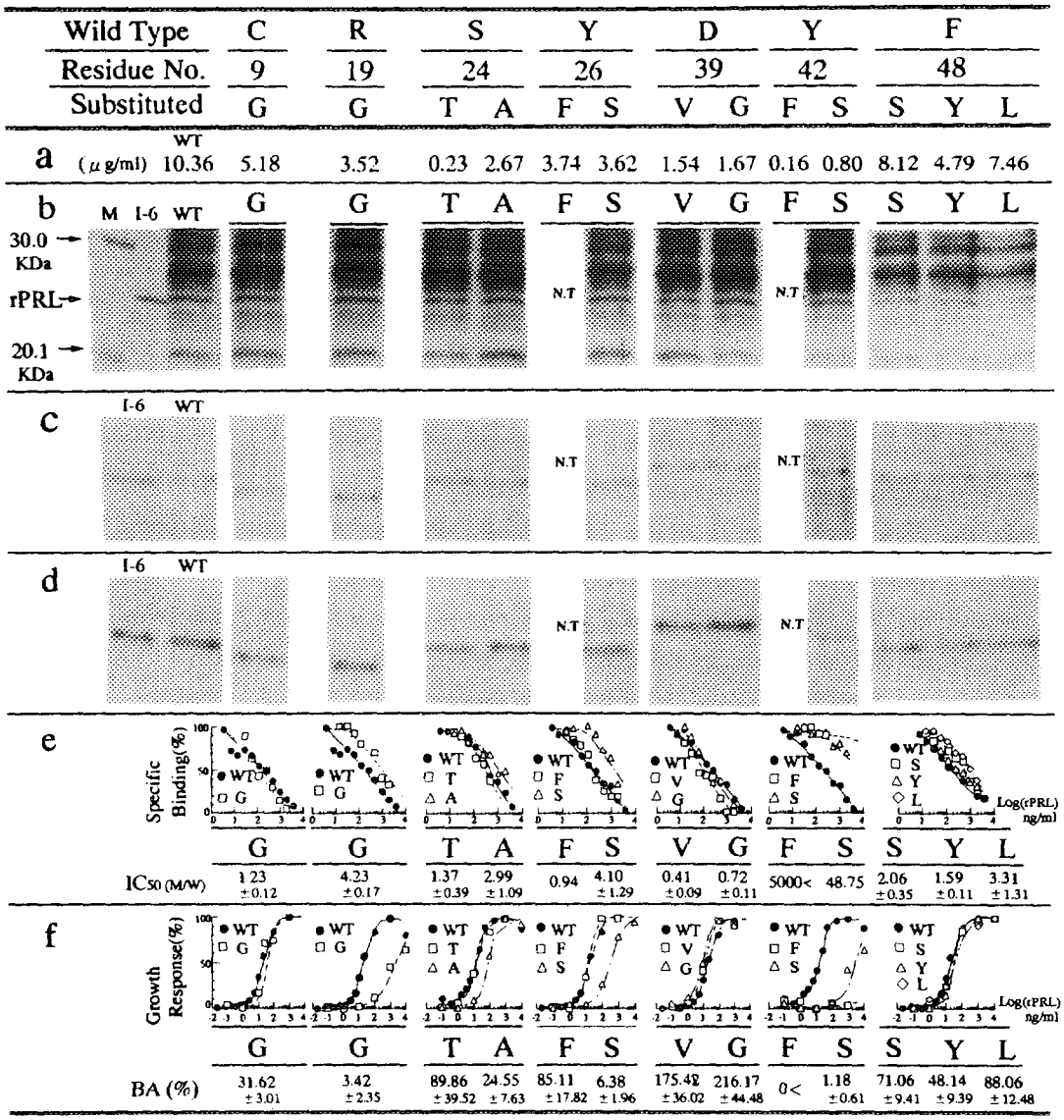

Fig. 2. Biochemical and biological analyses of mutant PRLs. The amounts of mutant PRLs expressed in COS-1 cells are indicated (a). Analyses by SDS-PAGE (b), non-denaturing PAGE (c), and Western blotting (d) were performed using the culture media, each containing about 0.2 μg PRL, as described in ***Materials and Methods.*** Radioreceptor (RRA, e) and Nb2 proliferation assays (Nb2, f) of mutant rPRLs were performed as described in ***Materials and Methods.*** The relative receptor binding inhibition (IC_{50}) was calculated as the ratio of the concentration that gave 50% activity with the mutant rPRL against that of recombinant wild type (WT) rPRL. The relative bioactivity (RA) by Nb2 proliferation assay was calculated as the ratio (%) of the concentration that gave 50% activity with the recombinant wild type (WT) rPRL against that of mutant rPRL. Molecular size marker is indicated by M. As controls, rPRL I-6 (I-6) and recombinant wild type rPRL (WT) were used. Data not obtained due to low concentrations are indicated as N.T.

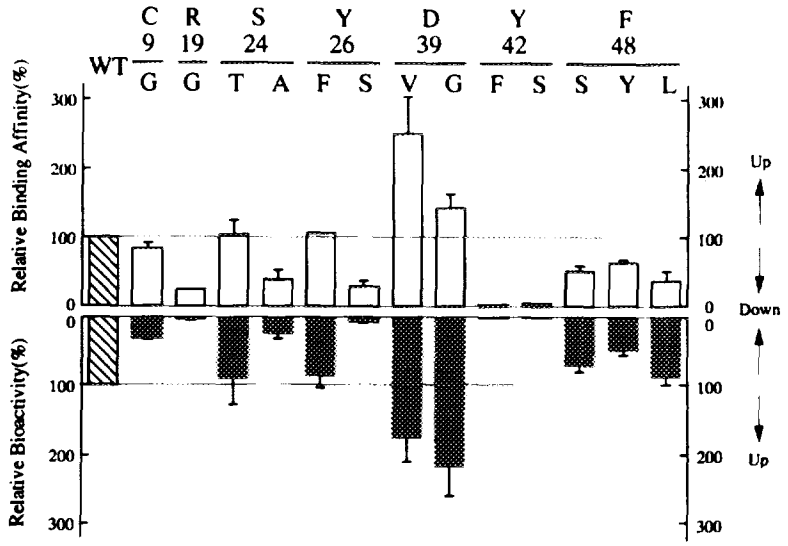

Fig. 3. Relative activities of receptor binding (RRA) and Nb2 proliferation (Nb2). The relative binding affinity (upper panel) was caluculated as inverse ratio (%) of IC_{50} in Fig. 2e. In the lower panel, RA in Fig. 2g is shown. The relative activities of RRA and Nb2 are shown in upper and lower panels. Error bars were calculated from repeat experiments (2~3),each with triplicate samples.

structure-function relationship of PRL is currently approached in reference to the three dimensional structure of GH (2). The importance of the amino acids in the highly conserved regions for the PRL activity has become recognized due to studies involving site-directed mutagenesis (4, 20, 21). However, there is little understanding of the amino terminal region, which is less evolutionarily conserved.

The results of this study demonstrated that some of the amino acids in the amino terminal region have a critical role for PRL activity. We obtained 13 mutants of 7 amino acid residues and the results for each are considered as follows. The first disulfide bond between residues 4 and 9 is not critical for the receptor binding, but its disruption causes a decrease in proliferation activity. This result is not consistent with a study of chemical modification (22). The alkylation of this disulfide bond did not change the activities on the mammary gland and pigeon crop sac bioassays, but enhanced the activity of the fish urinary bladder bioassay (22). The reason for this discrepancy is not known at present. But this residue may be involved in a species-specific function. The replacement of arginine 19 with glycine resulted in a decrease in the receptor binding (33%) and of cell growth activity (1%). Since Luck *et al.* (21) observed that the replacement of this residue with lysine retained about 87% of the proliferation activity, the positive charge at this position may be important for biological activity. The

mutation from serine at 24 to threonine (T) did not alter the activities, but replacement with alanine (A) decrease both activities to less than 33%. The hydroxyl group of this residue may be involved in the biological activity. Replacing tyrosine 26 with serine decreased the receptor binding and the proliferation activities to less than 25%, whereas a change to phenylalanine had no effect. The aromatic group may thus be important for the activities. Aspartic acid at 39 is not critical for the biological activities. A depletion of this negative charge would enhance the biological activity of rat prolactin. The tyrosine at 42 has a critical role for the receptor binding. Replacement with phenylalanine and serine abolished the biological activity, suggesting that the spatial configuration of the phenolic side chain is important. This residue is the most reactive among the nine tyrosines of PRL for iodination (23), suggesting that it is suitable located at the surface of PRL to interact with the receptor. Since amphibians and fishes have phenylalanine at this position (Fig. 1), it may perform a species specific role. The phenylalanine at position 48 is slightly involved in biological activity. Replacement with leucine decreased the receptor binding activity by 33%, but 88% of the proliferation activity was retained. A hydrophobic aliphatic side chain would decrease the binding but enhance a structural change of receptor to transduce intracellular signaling.

We found here that all the residues examined participate to some extent in receptor binding and proliferation activities. We demonstrated for the first time that the tyrosine at 42 has a critical role in the biological function of PRL. Though Luck *et al.* did not observe a significant residue involved in biological function (20, 21), our results show otherwise. Conversely, some of the amino acids in the highly conserved regions of central and carboxyl terminus are important for the PRL activity (4, 20, 21). PRL may play a biological role by using all the amino acids along its entire length to interact with its membrane receptor. However, the role of the conserved amino acids in the central and carboxyl terminal regions remains unknown. Further investigation of these residues will help define the relationships between the structure and function of PRL.

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