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Enhancement of the Receptor Binding and Nb2 Proliferation Activities of Rat Prolactin by Site-Directed Mutagenesis

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ABSTRACT—To investigate the roles of the amino acids of rat prolactin (rPRL), the structure of which is presumed to consist of an antiparallel, four-α-helix bundle, mutations constructed by site-directed mutagenesis were assayed in terms of their receptor binding and Nb2 cell proliferation activities. Replacement of P64L (replacing proline at position 64 with leucine) and K67E, which are located in the long loop region between helices 1 and 2, produced drastic decreases in the binding and proliferation activities. Mutations at D91 and E118 in the second and third helices, respectively, resulted in increased Nb2 cell proliferation activity with a slight increase in receptor binding activity. Mutations at L81 in the second helix and Y145 and W148 in the second loop between helices 3 and 4 produced no marked changes. Mutation at D158N in helix 4 markedly increased receptor binding activity with a slight loss of Nb2 cell proliferation activity, although two other mutations, D158H and D158R, produced a decrease of receptor binding activity without notable changes in Nb2 cell proliferation activity. These results demonstrate that P64 and K67 are crucial for PRL function while replacement of D91, E118 and D158 possibly leads to functional enhancement.

INTRODUCTION

Prolactin (PRL) is an anterior pituitary hormone that exhibits several functions in a wide range of vertebrates by binding to specific membrane receptors (Nicoll *et al.*, 1986). The three-dimensional structure of growth hormone (GH) has been determined (Abdel-Meguid *et al.*, 1987; de Vos *et al.*, 1992), and the basic structure of PRL is presumed to be an antiparallel, four-α-helix bundle because of the structural and genetical relationships between these two hormones. Recently, Goffin *et al.* (1994) reported the presence of two receptor binding sites on each PRL molecule, as is the case with GH (Fuh *et al.*, 1992). The first binding site is formed by residues on the exposed faces of helices 1 and 4 and by the residues in the long connecting loop between helices 1 and 2. The second binding site is formed by the exposed sides of helices 1 and 3. Genetic engineering has been performed on PRL, and the amino acids critical for PRL function have been found in helix 1 (Luck *et al.*, 1989, 1991; Maruyama *et al.*, 1994), the connecting region between helices 1 and 2 (Goffin *et al.*, 1992) and helix 4 (Goffin *et al.*, 1993, 1994; Kato *et al.*, 1996). We previously found that the amino acids in helix 1 are essential for rPRL function (Maruyama *et al.*, 1994) and that helix 4 of rPRL plays a critical role, as does helix 4 of hGH, when it binds to the PRL receptor rather than to the GH receptor (Kato *et al.*, 1996).

Though the molecular bases of the various effects of PRL remain elusive, several recent studies have revealed a new aspect of its function. PRL is known to promote proliferation of a lymphocyte lineage cell line, Nb2 (Tanaka *et al.*, 1980). Several investigators have demonstrated that PRL stimulates lymphocyte proliferation by activating a protein tyrosine kinase, designated $p59^{fyn}$, which is associated with the PRL receptor (Clevenger and Medaglia, 1994; McMurray *et al.*, 1992). Analyses of the amino acids crucial for PRL function will help to clarify its structure-function relationships as well as the diversity of its many functions at the molecular level.

In this study the contributions to PRL function of certain amino acids that have not been investigated previously (Kato *et al.*, 1996; Maruyama *et al.*, 1994) were examined in terms of receptor binding and Nb2 proliferation activities (Maruyama *et al.*, 1994; Shimokawa *et al.*, 1990). The results demonstrated that replacement of P64 and K67 affected PRL receptor binding while changes at D91 and E118 enhanced Nb2 proliferation activity.

MATERIALS AND METHODS

Vector construction, mutagenesis and expression

Construction of the clones containing rPRL (rPRLSG) and site-directed mutagenesis were performed as described previously (Shimokawa *et al.*, 1990).

Mutated clones were transfected with COS-1 cells (supplied by Japanese Cancer Research Resources) using 200 μl DNA/DEAE-dextran at 2 μg DNA/ 5×10^5 cells / 60 mm dish for 15 min (Luthman and Magnusson, 1983). PRLs were expressed in 3 ml serum-free GIT medium (Wako Pure Chemicals, Osaka, Japan). The culture medium was exchanged every 3-4 days, and the conditioned medium was collected each time. The media for each clone were combined

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and stored without purification until further analysis, as described previously (Maruyama *et al.*, 1994; Shimokawa *et al.*, 1990).

PRL quantification and Western blotting

PRL was quantified by radioimmunoassay (RIA) and competitive binding assays were performed using the double antibody method, employing a NIADDK RIA kit consisting of rPRL I-6, anti-rPRL S-9 and rPRL RP-3, as described previously (Maruyama *et al.*, 1994). The second antibody was goat anti-rabbit IgG serum, HAC-RBA2-03GTP86, raised by Dr. K. Wakabayashi at the Biosignal Research Center of the Institute for Molecular and Cellular Regulation, Gunma University.

Electrophoresis on 12.5% w/v sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and 12.5% w/v non-denaturing polyacrylamide gel (non-denaturing PAGE) was carried out as described by Laemmli (1970), and Orstein (1964) and Davis (1964), respectively, followed by staining with Coomassie brilliant blue or electroblotting onto a nitrocellulose membrane. For mutations which did not exhibit a parallel curve in the competitive binding assays described above, quantification was carried out by staining after separation by non-denaturing PAGE using wild type PRL (WT-PRL) as a control. In this analysis, expression of the pSG5 vector itself yielded no band at the position corresponding to the WT and mutant rPRLs, and there was no polymerized PRL. Proteins were Western-blotted by incubating the membrane with rabbit anti-rPRL S-9, followed by horseradish peroxidase-conjugated second goat anti-rabbit IgG antibody, and the color was developed using H_2O_2 and horseradish peroxidase reagent (Konica, Tokyo, Japan).

Radio-receptor-binding assay (RRA)

The RRA was performed as described previously (Goffin *et al.*, 1992). Rat PRL I-6 was labeled with ^{125}I using lactoperoxidase (Miyachi *et al.*, 1972). Competitive binding in pregnant rat liver microsomes (100 μg) was measured using ^{125}I-rPRL (about 1×10^5 cpm) in the presence of recombinant rPRL followed by overnight incubation at 25°C. The precipitates were collected by centrifugation at 11,000 × g and their radioactivity levels were measured. The larger volumes of the medium with low PRL expression levels did not influence these results or those of the Nb2 proliferation assay.

Nb2 cell proliferation activity

The proliferation activity of Nb2 node lymphoma cells was measured colorimetrically in Fischer's medium (GIBCO, Gaithersburg, MA) containing 10% v/v FCS using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) method (Mosmann, 1983) as described previously (Maruyama *et al.*, 1994).

RESULTS

Biochemical and immunological analyses

Figure 1 shows the sequence homology among PRLs from several different animals. The amino acids conserved throughout evolution are present mainly in helices 1 and 4 and in the long loop region between helices 1 and 2. In this study, site-directed mutagenesis was carried out on 10 amino acids located between helices 1 and 4 as shown in Table 1. The 12 primers designed to mutate the amino acids which are conserved at least in tetrapods are listed in Table 1, and 15 mutant PRLs with altered amino acid residues at 9 positions (Fig. 1), except for D66, were obtained. Quantitative RIA showed that most of these mutations yielded competitive binding curves parallel to that of recombinant WT-PRL, but P64L (proline at position 64 replaced with leucine) gave a slightly shifted curve (data not shown), indicating that a partial change in antigenicity had occurred. The amounts of mutant PRLs expressed (Table 1) varied from less than 0.01 to 21.9 μg/ml. The W148C mutant expressed a consistently low level (below 0.01 μg/ml) in repeated experiments (data not shown).

SDS-PAGE and Western blotting analyses of the mutant PRLs (data not shown), except for a few samples with very low expression levels, showed that their molecular masses (23 kDa) were the same as that of WT-PRL. When the same samples were separated by non-denaturing PAGE followed by Western blot analysis, the K67E, E68K, D91G, E118Q, D158N, D158H and D158R mutants showed different mobilities from that of WT-PRL. Most of the mobility changes corresponded to a change in the net protein charge due to amino acid replacement. Adequate volumes of mutants E118K and W148C, which showed low expression levels when

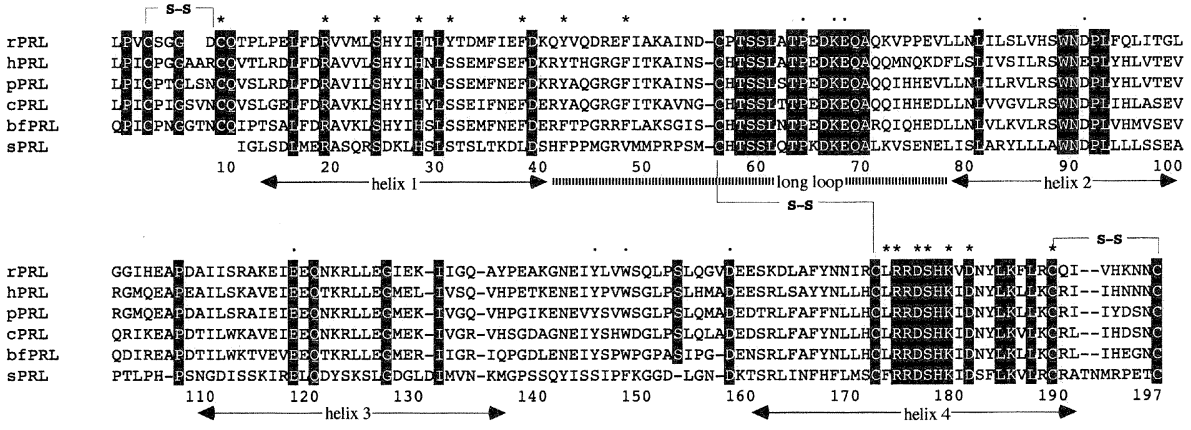


Fig. 1. Amino acid sequence of PRL. The amino acids of rPRL are compared with those of porcine (Kato *et al.*, 1989), human (Cooke and Baxter, 1982), chick (Hanks *et al.*, 1989), bullfrog (Takahashi *et al.*, 1990) and salmon (Song *et al.*, 1988) PRLs. The amino acids conserved throughout evolution are indicated by white letters and gaps (open spaces) have been introduced to allow maximum homology to be indicated. The amino acids replaced in this study are indicated by (•). The amino acids examined in previous studies (Kato *et al.*, 1996; Maruyama *et al.*, 1994) are indicated by (*).

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estimated by RIA, were analyzed by SDS-PAGE and non-denaturing PAGE, but no bands corresponding to rPRL were found, suggesting that they indeed expressed very low levels of PRL.

Receptor binding

The competitive receptor binding of mutant PRLs, except for W148C, to the liver membrane fraction from pregnant rats was measured (Fig. 2). In order to quantify the results, the receptor binding affinity (RBA_{50}) was calculated as the ratio of the concentration of mutant PRL that reduced the binding of WT-PRL by 50%, while the relative receptor binding affinity ($RRBA_{50}$) was calculated as the inverse ratio (%) of the RBA_{50} (Fig. 3, upper panel).

Binding of mutant P64L to the receptor decreased activity drastically, as well as altering the antigenicity. Changing the positive charge to a negative one at residue K67 (proximal to residue P64) also reduced activity by about 90%. In contrast,

Table 1. Oligonucleotide primers used to mutate rat prolactin

Residue Number	Change of Amino Acid		Oligonucleotide	PRL Expressed (μg/ml)
	from	to		
64	P	**L**	AGCTACTC(T)TGAAGACAAGGAACAAGCC	21.9 ± 4.3
66	D	N	GCTACTCCTGAA(A)ACAAGGAACAAGCC	–
67	K	**E**	GCTACTCCTGAAGAC(G)AGGAACAAGCC	20.4
67・68	K・E	**A・A**	CTCCTGAAGAC(G)(C)GG(C)ACAAGCCCAGAA	21.4
68	E	Q/**K**	AAGACAAG(C/A)AACAAGCC	– /3.7
81	L	**V**/A	CTTTTGAAC(G)(T/C)GATCCTCAGTTT	16.1 / –
91	D	**G**/A/V	CCTGGAATG(G/C/T)CCCTCTGT	14.6 / – / –
118	E	**Q**/**K**	AAGAGATT(C/A)AGGAACAA	21.2 ± 4.3 / 0.189
145	Y	**S**/F	TGAGATCT(T/C)CTTGGTTTGGTCACAACTC	20.8
148	W	**F**/**L**/**C**	GATCTACTTGGTTT(T/G)(T/G)TCACAACTC	12.8 / 8.5 / 0.004
158	D	H/**N**	AAGGAGTT(C/A)ATGAAGAA	– /9.7
158	D	**R**/**H**	AAGGAGTT(C)(G/A)TGAAGAATCC	14.5 / 9.1

Mutations designed to replace amino acids at two positions are indicated by (・). The mutated nucleotides are indicated in parentheses and mixed nucleotides at a single point are indicated by a slash. Replacement amino acids which successfully mutated are indicated by large bold capitals.

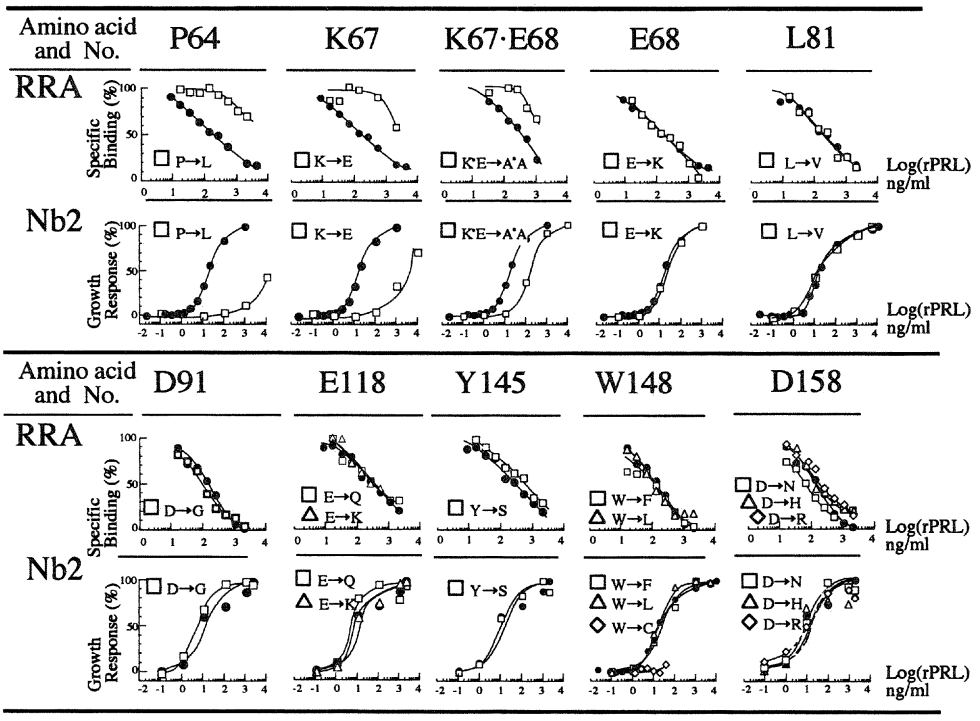


Fig. 2. Biological analyses of mutant PRLs. Radioreceptor assays (RRA) of mutant rPRLs were performed as described in Materials and Methods. The control was recombinant WT-PRL, the data for which are indicated by solid circles. The Nb2 proliferation activity (Nb2) of mutant rPRLs was assayed as described in Materials and Methods.

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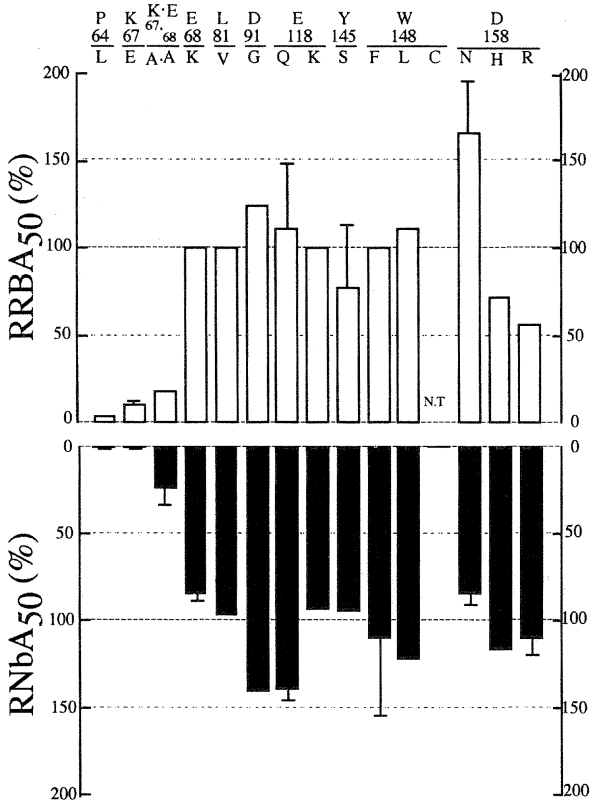


Fig. 3. Relative receptor binding ($RRBA_{50}$) and Nb2 proliferation ($RNbA_{50}$) activities. The relative receptor binding ($RRBA_{50}$) and Nb2 proliferation ($RNbA_{50}$) activities are shown in the upper and lower panels, respectively. The $RRBA_{50}$ was calculated as the inverse ratio (%) of the RBA_{50} shown in Fig. 2. In the lower panel, $RNbA_{50}$ values calculated from Fig. 2 are shown. Error bars were determined from 2~3 repeat experiments on triplicate samples. Data not obtained due to extremely low concentrations are indicated as N.T.

replacing the next (E68) negative charge with a positive one did not change activity. Replacing two amino acids (K67 and E68) with a neutral residue, alanine (A), reduced activity to a level similar to that of K67E. L81, D91, E118, Y145 and W148 had only a small effect on receptor binding activity, whereas slight increases (20-25%) were observed with D91G and Y145S. Mutating the residue at D158 with three different amino acids produced varied results: D158H and D158R reduced receptor binding activity to about 55~70%, whereas D158N increased it to about 170%.

Nb2 proliferation activity

The biological activities of recombinant rPRLs were measured by examining their effects on the proliferation of rat lymphoma Nb2 cells, as the recombinant rPRL expressed in mammalian cells has activity equivalent to that of pituitary-derived rPRL (Shimokawa *et al.*, 1990). Figure 2 shows the results of the Nb2 proliferation activity assay in the presence of increasing amounts of mutant PRLs. The relative Nb2 activity ($RNbA_{50}$) was calculated as the ratio (%) of the WT-PRL concentration that showed 50% activity compared with that of mutant rPRL (Fig. 3).

Mutants P64L and K67E exhibited no Nb2 proliferation activity. Mutant E68K showed 90% activity while dual replacement at positions 67 and 68 (K67A·K68A) resulted in low proliferation activity, a similar situation to that observed in the receptor binding assay. Mutations between residues 81 and 148 did not decrease Nb2 proliferation activity, except for mutants D91G and E118Q, which showed an increase. W148C (0.1~40 ng/ml medium) showed no activity. Mutant D158N induced Nb2 proliferation to about 85% of the control level despite increasing receptor binding about 1.7 fold.

DISCUSSION

We have previously reported that a considerable number of amino acids in the amino terminal region (helix 1), the loop region between helices 1 and 2, and the carboxy terminal region (helix 4) play a critical role in PRL function (Kato *et al.*, 1996; Maruyama *et al.*, 1994). The present study comprehensively demonstrated that amino acids (P64 and K67) in the loop region between helices 1 and 2 are crucial for PRL function and that replacement of certain other amino acids (D91, E118 and D158) results in functional enhancement.

Since PRL was first purified, several investigators have attempted to clarify its structure-function relationships by means of chemical modification and genetic engineering. However, chemical modifications of PRL yielded only the information that residue Y42 is chemically reactive (Kawauchi *et al.*, 1977) and that the disulfide bond between residues 56 and 172 may be critically important for the molecular folding that produces the biologically active form (Doneen *et al.*, 1979). In contrast, site-directed mutagenesis has so far generated much specific information. Consequently, the importance of amino acids R19, Y26, C56, P64, K67, R174, R175, K179 and K185 for PRL function has been clarified (Goffin *et al.*, 1992, 1993, 1994; Kato *et al.*, 1996; Maruyama *et al.*, 1994), though information regarding the others is still lacking. The tertiary structure of PRL is currently presumed to consist of an antiparallel four-α-helix bundle, similar to that of GH (Cunningham *et al.*, 1990; Cunningham and Wells, 1991), though there may be slight topological differences in the contributions of amino acids in the loop region between helices 1 and 2 (Goffin *et al.*, 1992). In the structure, two binding sites with almost equivalent affinity for the PRL receptor are present. The roles of amino acids in helices 1 and 4 and the loop region between helices 1 and 2, which may form binding site 1 (de Vos *et al.*, 1992), have been well characterized by several workers (Goffin *et al.*, 1992, 1993, 1994; Kato *et al.*, 1996; Luck *et al.*, 1989, 1991; Maruyama *et al.*, 1994). In contrast, binding site 2 (de Vos *et al.*, 1992), probably formed by the fronts of helices 1 and 3, has been less fully characterized. Our previous results revealed the contribution of few amino acids in helix 1 to be binding site 2 (Maruyama *et al.*, 1994). In addition, the present study showed that E118, which is located in helix 3 and binding site 2, did not have a critical role in PRL

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function. It is noteworthy that mutant E118Q increased Nb2 proliferation activity, whereas another mutation, E118K, did not cause any change in either activity, suggesting that neutralization of the negative charge activates intracellular signaling to promote Nb2 cell proliferation.

Goffin *et al.* (1992) replaced the P64 residue with alanine (P64A) and, using circular dichroism, demonstrated that the three-dimensional structure remained unchanged, but that PRL function decreased markedly. The present study also found that replacement of P64 with leucine (P64L) lowered the antigenicity of PRL, presumably by interfering slightly with its three-dimensional structure, since proline is known to be linked to neighboring amino acids by a *cis*-peptide bond that forms a rigid configuration with the adjacent residues. In addition, mutation at K67 dramatically reduced receptor binding and Nb2 proliferation activities. Therefore, one explanation for the loss of activity due to mutation at position P64 is that a change of the *cis*-peptide may induce formation of a distinct configuration with adjacent residues, especially K67, whose positively charged side group is critical for binding to the PRL receptor.

Some semi-conserved and conserved amino acids are present among the residues between positions 71 and 171 (Fig. 1). However, replacing some of these amino acids demonstrated that most of them are not critical for PRL function. It is noteworthy that D158N increased the receptor binding activity by 1.7 fold, whereas its Nb2 proliferation activity was decreased slightly. In addition, replacement at two acidic residues (D91 and E118) increased Nb2 proliferation activity without any marked change in the receptor binding activity. Thus, there are no critical amino acids in positions that could affect receptor binding and/or proliferation activities in this region except for D91 and E118. Mutation to increase PRL function might be valuable in producing highly potent PRL as an antagonist or agonist.

The amino acids that exhibited a valid contribution to PRL function were considered to be located in the presumed tertiary structure of PRL (Fig. 4), based on reference to the crystallographic structure of GH (Abdel-Meguid *et al.*, 1987; de Vos *et al.*, 1992). D91 and E118 in helices 2 and 3 are located close to the other helices, suggesting that the loss of negative charge results in interactions with neighboring residues in other helices to strengthen Nb2 proliferation activity. On the other hand, replacement of D158 with three other amino acids resulted in varying activities. This residue is located at the beginning of helix 4 and close to the end of helix 1, suggesting that D158 plays a role in determining the configuration of the four-α-helix bundle. Mutation of W148C resulted in loss of PRL function, whereas other mutations of W148 did not produce marked changes. The introduction of a cysteinyl residue might produce a disorder of disulfide formation. It is noteworthy that one of the three disulfide bonds in wild type PRL is critical for PRL function (Doneen *et al.*, 1979).

Our present study has comprehensively demonstrated that certain amino acids in the amino terminal (helix 1), the long loop between helices 1 and 2, and helix 4 play a crucial role in PRL function. These findings have revealed the supplemental roles of certain residues (D91, E118, D158) in PRL function, as well as confirming the importance of P64 and K67. Mutations which increase one activity without marked loss of the other suggest the possibility that PRL antagonists and/or agonists can be developed.

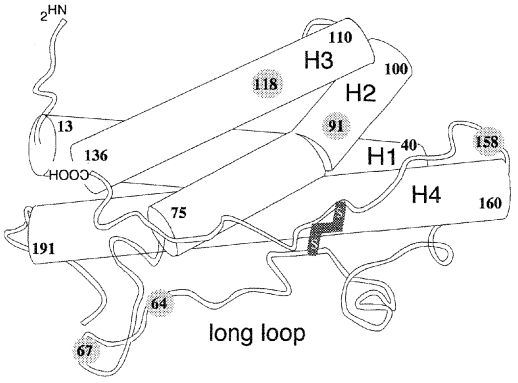


Fig.4. Approximate location of residues predicted for rPRL in the present study. The structural model was based on previous reports (Abdel-Meguid *et al.*, 1987; Cunningham *et al.*, 1989). The helical start and end points are numbered according to the corresponding numbers for PRL by referring to the homology between PRL and GH.

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