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Increased Thermo-Stability of Rat Prolactin after Replacing Glutamic Acid at Position 118 by Lysine

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ABSTRACT—We examined the structural stabilities after heat treatment of 22 mutants of rat prolactin (rPRL) with amino acid replacements at 15 different positions and recombinant wild-type rPRL (WT-PRL) as part of our series of studies on site-directed mutagenesis of rPRL. When WT-PRL at low concentrations (0.1~10 ng/ml) was heated at 100°C for 20 min, it lost its Nb2 proliferation activity, whereas at high concentrations (above 1 μg/ml), its activity remained. Temperature-dependent loss of the proliferation activity of 10 ng/ml WT-PRL after heat treatment for 5 min was observed. Next, we examined the proliferation activities of the 22 mutants heated at 60 and 70°C for 5 min. After treatment at 60°C, all the mutants retained their initial proliferation activities, whereas treatment at 70°C reduced their activities to about 63%, except for one in which glutamic acid at position 118 was replaced by lysine (E118K), suggesting that the mutations did not induce structural instability. The mutant E118K retained 84% of its initial activity after treatment at 70°C, significantly ($P < 0.01$) higher than the WT-PRL value. The temperature-dependency profile of the Nb2 proliferation activity of E118K also showed it had significantly increased thermo-stability. Meanwhile another mutant (E118Q) at the same residue showed no increased thermo-stability, suggesting that changing a negative charge (E) to a positive one (K) at position 118 induces ionic bond formation with a neighboring negative charge, resulting in thermo-stabilization of the structure of PRL.

INTRODUCTION

Prolactin (PRL) is an anterior pituitary hormone that has several functions in a wide range of vertebrates (Bern and Nicoll, 1968). An antiparallel, four-α-helix bundle is believed to be a basic structure of PRL by referring to the three-dimensional structure of growth hormone (GH), which has been determined (Abdel-Meguid *et al.*, 1987; de Vos *et al.*, 1992), since PRL and GH share the same ancestral gene. We have carried out comprehensive investigations into PRL using site-directed mutagenesis and found that amino acids in helices 1 and 4 and in the long loop between helices 1 and 2 are important for its function (Kato *et al.*, 1996; Maruyama *et al.*, 1994). Our recent study revealed that the important amino acids in the fourth helix of PRL contribute in the same manner as those of human GH (hGH) when it binds to the PRL, not the GH, receptor (Kato *et al.*, 1996). Thus, taken together with the results of investigations conducted in other laboratories (Goffin *et al.*, 1992, 1993, 1994; Luck *et al.*, 1989, 1991; Maruyama *et al.*, 1994), our knowledge about the biological roles of each amino acid in PRL is accumulating. PRL is known to promote proliferation of the lymphocyte lineage cell line Nb2. Recently, several investigators demonstrated that PRL stimulates lymphocyte proliferation by activating protein tyrosine kinase $p59^{fyn}$, which is associated with the PRL receptor (Bernton *et al.*, 1988; Clevenger *et al.*, 1990; Clevenger and Medaglia, 1994; Hartmann *et al.*, 1989; McMurray *et al.*, 1992; Pellegrini *et al.*, 1992). Further studies on the biological roles of its amino acids should elucidate the various PRL functions at the molecular level and lead to the development of approaches to study the biological and clinical applications of this hormone.

In this study, we examined whether replacing of amino acids would alter the thermo-stability of PRL, as a notable characteristic of ovine PRL is that it does not lose its biological activity after heat treatment at 100°C for 20 min (Li, 1980), providing a useful parameter to evaluate the effects of mutations. The wild type of rat PRL (WT-PRL) and most of its mutants showed reduced Nb2 proliferation activity to a similar extent after heat treatment. One exception was the mutant E118K (glutamic acid at position 118 was replaced by lysine), which exhibited 50% of the initial Nb2 proliferation activity of WT-PRL at about 82°C that was 7°C higher than that of WT-PRL, suggesting that changing from a negative charge (E) to a positive one (K) increases the thermo-stability of PRL by altering the intramolecular interaction as a result of ionic bond formation.

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MATERIALS AND METHODS

Vector construction, mutagenesis and expression

Construction of clones containing rPRL (rPRLSG) and site-directed mutagenesis were performed as described previously (Maruyama *et al.*, 1994; Shimokawa *et al.*, 1990). PRLs obtained from the serum-free GIT culture media (Wako Pure Chemicals, Osaka, Japan) were used and each PRL was characterized as described previously (Kato *et al.*, 1996; Maruyama *et al.*, 1994).

Heat treatment of rPRL

rPRLs, at the required concentrations, in serum-free GIT media indicated were heated at several temperatures (45~100°C indicated) for 5 or 20 min and then chilled quickly on ice until assayed. In order to compare their stabilities at 60 and 70°C, 22 mutants with amino acid replacements at 15 positions, at concentrations ranging from 4.6 to 256.4 ng/ml that gave the same biological activity as that of WT-PRL (10 ng/ml), were heated for 5 min.

Nb2 cell proliferation activity

The Nb2 node lymphoma cells (a gift from Dr. T. Tanaka) were placed in 96-well culture plates at 1×10^4 cells/100 μl/well, as described by Shimokawa *et al.* (1990), and 3.3 μl of rPRLs at the required concentration, was added to each well and incubated at 37°C in an atmosphere of 95% air-5% CO_2 for 72 hr, aftetr which cell growth was measured colorimetrically using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) method (Mosmann, 1983), as described previously (Maruyama *et al.*, 1994). The value, $Nb2^{50}$, was obtained the activity where the PRL exhibited 50% of the initial Nb2 proliferation activity of each unheated sample.

Statistical analysis

The results are presented as means ± SE of triplicate samples in three independent experiments and analyzed using Student's *t*-test. Differences at $P < 0.05$ were considered significant.

RESULTS

The stability of rPRL after heat treatment determined by the Nb2 proliferation assay

We reported that rPRL I-6 preparations from pituitary and WT-PRL have equivalent biological activities in the Nb2 proliferation and receptor binding assays (Shimokawa *et al.*, 1990). Figure 1A shows the Nb2 proliferation activity of WT-PRL, at various concentrations, after treatment at 100°C for 20 min. WT-PRL not subjected to heat treatment showed Nb2 proliferation activity at a concentration as low as 1 ng/ml, but heat treatment of WT-PRL at low concentrations (0.1~10 ng/ml) resulted in the loss of most of its biological activity, although at 100 ng/ml, slight activity was retained and above 1 μg/ml, 80% of its activity remained. Thus, the heat-induced loss of activity depended on the concentration of WT-PRL.

To evaluate the loss of activity by increasing the temperature, WT-PRL at 10 ng/ml WT-PRL was heated at various temperatures, indicated in Fig. 1B, for 5 min. WT-PRL treated at 50°C showed slightly increased activity and it retained its initial activity up to 60°C, whereas treatment at temperatures above 60°C reduced its activity rapidly.

Effect of amino acid replacement in rPRL

As the initial Nb2 proliferation activity of WT-PRL was retained up to 60°C and decreased to 63% after treatment at 70°C, the activities of the mutant rPRLs were examined after treatment at both temperatures for 5 min (Fig. 2). The concentration of each mutant rPRL was adjusted to produce the same activity as 10 ng/ml WT-PRL. As shown in Fig. 2, their activities were mostly retained after treatment at 60°C.

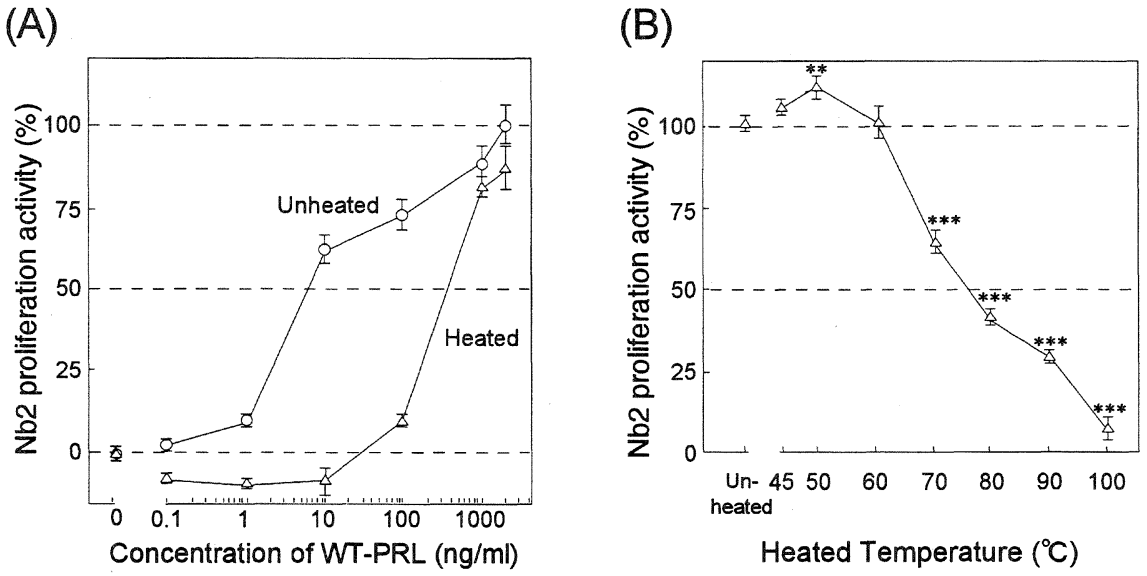

Fig. 1. Nb2 proliferation activity of rat prolactin (rPRL) after heat treatment. Wild type rPRL (WT-PRL), at the concentrations indicated, was heated at 100°C for 20 min and then the Nb2 proliferation activity was measured and expressed as a ratio (%) of that of 2 μg/ml unheated WT-PRL (A). WT-PRL (10 ng/ml) was heated for 5 min at the temperatures indicated and then the Nb2 proliferation activity was measured and expressed as the ratio (%) of that of 10 ng/ml unheated WT-PRL, which was taken as 100%. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the activity of unheated WT-PRL (B).

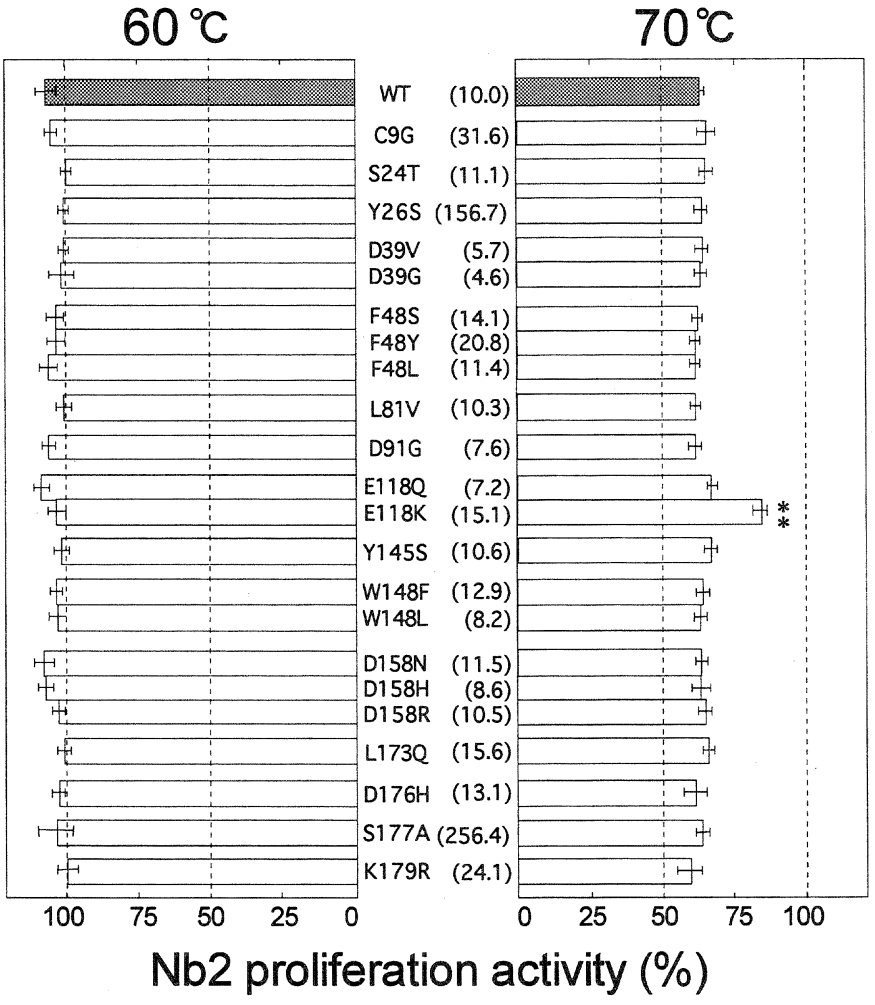

Fig. 2. Nb2 proliferation activities of 22 mutants of rPRL. Twenty two mutants with amino acid replacements at 15 positions, such as C9G, a mutant with the cystine at residue 9 replaced by glycine, were tested. The concentration of each mutant to be heated, indicated in parentheses, was adjusted so its bioactivity was equal to that of 10 ng/ml WT-PRL, as described previously (Kato *et al.*, 1996; Kato *et al.*, unpublished; Maruyama *et al.*, 1994). The Nb2 proliferation activity is expressed as a ratio (%) of that of each unheated mutant, which was taken as 100%. $^{**}P < 0.01$ *vs.* the activity of WT-PRL at each temperature.

In contrast, treatment at 70°C reduced their activities, with one exception, E118K. As higher concentrations of mutants Y26S and S177A than the others were heated, they showed a similar decreases in the activity. The mutant E118K still retained 84% of its activity after treatment at 70°C and this was significant compared with WT-PRL ($P < 0.001$), indicating that replacement of glutamic acid at position 118 by lysine increased the thermo-stability of WT-PRL.

In order to obtain the temperature-dependency profile of Nb2 proliferation activity, the mutant E118K was heated at increasing temperatures, as were WT-PRL and the E118Q cognate mutant of E118K. As shown in Fig. 3, the profile of E118Q showed it had slightly higher activity than WT-PRL, but this failed to reached significance. However, the profile of E118K showed it had higher activity than WT-PRL. The apparent differences between the profiles of E118K and WT-PRL suggest that replacing glutamic acid at position 118 with lysine was responsible for the stability during heat treatment.

DISCUSSION

We have carried out comprehensive studies of the amino acids important for the biological functions of PRL using site-directed mutagenesis and several amino acids critical for such functions were found in the semi-conservative amino acid terminal region (Maruyama *et al.*, 1994). A cluster of important amino acids is present in the highly conserved carboxyl terminal region forming the fourth helix, and these amino acids are presumably involved in PRL binding to the PRL receptor, in a topologically similar manner to the binding of hGH to the PRL receptor, rather than the GH receptor (Kato *et al.*, 1996). On the other hand, amino acids in the second and third helices and the intervening region between helices 3 and 4 were found not to be concerned with the Nb2 proliferation activity, except for the enhanced receptor binding activity of D158N and increased Nb2 proliferation activities of D91G and E118Q compared with WT-PRL (Kato *et al.*, unpublished). These

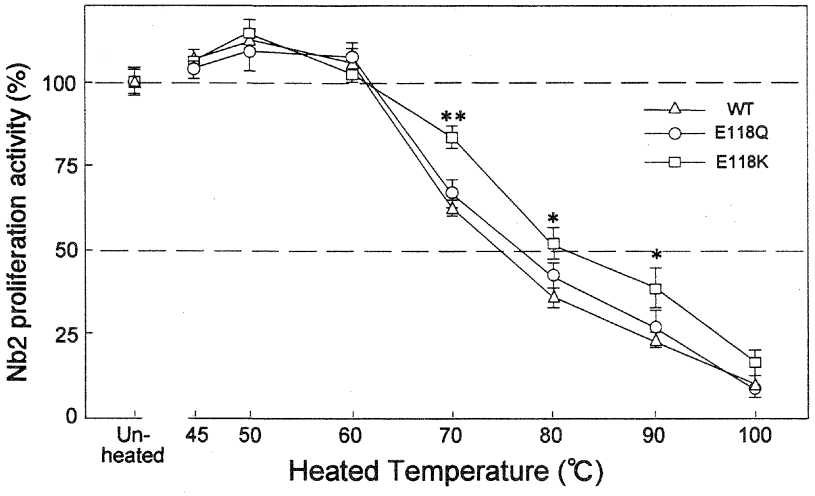

Fig. 3. Temperature-dependent reductions of the Nb2 proliferation activities of WT-PRL and its E118 mutants. WT-PRL, E118Q and E118K, at concentrations of 10, 7.2 and 10.7 ng/ml, respectively, were heated at the temperatures indicated for 5 min. Nb2 proliferation activity is expressed as a ratio (%) of that of each unheated sample, which was taken as 100%. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the activity of WT-PRL at each temperature.

observations suggest novel aspects of PRL that may merit further research.

Our series of studies yielded 22 mutants with amino acid replacement at 15 positions that still have measurable activity for assaying PRL function. In this study, we evaluated the roles of these amino acids in thermo-stability by subjecting the mutants to heat treatment, as thermo-stability was mentioned by Li (1980) during the early stages of PRL research. They reported that ovine PRL was highly stable after treatment at 100°C for 20 min as well as under alkaline and acid conditions. In fact, heated rPRL showed this stability only at high concentrations, such as 1 μg/ml (Fig. 1A) and it lost its activity rapidly at concentrations below 100 ng/ml, suggesting a loss of activity in concentration-dependent manner (Fig. 1A). Temperature-dependent loss of Nb2 proliferation of WT-PRL was well produced at low concentration (10 ng/ml) as shown in Fig. 1B. Therefore, the concentrations of the mutant rPRLs examined were adjusted to 4.6 to 258.4 ng/ml, to give the same Nb2 proliferation activity as 10 ng/ml WT-PRL.

Mutations at C9, Y26, D39, F48, D91, D158, D176, S177 and K179 induced changes in receptor binding and/or Nb2 proliferation activities (Kato *et al.*, 1996; Kato *et al.*, unpublished; Maruyama *et al.*, 1994). Nevertheless, our present results suggest that serious structural damage resulting from heat treatment did not occur after these amino acid replacements. Repeated experiments demonstrated that most of the mutants showed the same Nb2 proliferation activity profile as WT-PRL (Fig. 2). Only one exception was found: the mutant E118K showed higher activity than all the others. When the thermo-stability profile was examined by progressively increasing temperature (Fig. 3), the $Nb2^{50}$ of E118K occurred at 82°C, whereas that of WT-PRL was 75°C. On the other hand, E118Q, which was mutated at the same position as E118K, showed no significant increase in thermo-stability. Therefore, the increased $Nb2^{50}$ value of E118K may correlate with the character of the side residue of the amino acid, especially the change from a negative charge (E) to a positive one (K). An alternative explanation is that introduction of a positive charge leads to formation of an ionic bond with a negatively charged residue nearby the residue at position 118. It is noteworthy that E118 is conserved in all vertebrates and replacing this residue with lysine changed neither the Nb2 proliferation activity nor the receptor binding activity (Kato *et al.*, unpublished).

In conclusion, this study showed that PRL had concentration-dependent thermo-stability and that replacing the amino acid residue at position 118 to change the negative charge (E) to a positive charge (K) increased the thermo-stability. None of the amino acids examined in this study are involved or play an important role in folding of the PRL structure.

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