Roles of Transmembrane Prolines and Proline-induced Kinks of the Lutropin/Choriogonadotropin Receptor*

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The lutropin/choriogonadotropin receptor is a seven-helix transmembrane (TM) receptor. A unique feature of TM helices is the content of Pro, which generally is absent in α helices of globular proteins. Because Pro disrupts helices and introduces a −26° kink, it has been speculated that Pro plays a crucial role in the structure of TM helices, exoloops, and cytoloops of TM receptors. To examine the roles of the five TM Pros of the lutropin/choriogonadotropin receptor, these residues were individually substituted. Mutant receptors were examined for surface expression, hormone binding, and cAMP induction. Surface expression was monitored after introducing the flag epitope into the receptors. Flag epitopes slightly affected cAMP induction but not hormone binding or surface expression of receptors as monitored by immunofluorescence microscopy and 125I-anti-flag antibody. The results indicate that Pro 479 in TM 4 and Pro 598 in TM 7 play important yet contrasting roles. Pro 479 is crucial for hormone binding at the cell surface but not after solubilization of the receptor. This is more likely due to the Pro side chain than the Pro-induced kink. Pro 598 is important for surface expression. The kinks of Pro 463 of TM 4, Pro 562 of TM 6, or Pro 591 of TM 7 are not important because the substitution of Phe for these residues did not significantly impact surface expression, hormone binding, and cAMP induction.

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§ The abbreviations used are: LH/CG receptor; LH, luteinizing hormone (lutropin); CG, chorionic gonadotropin (choriogonadotropin); TM, transmembrane; hCG, human chorionic gonadotropin; LH/CG-R, PBS, phosphate-buffered saline.
FIG. 1. **TM prolines and their conservation.** The location of TM Pros is shown in the putative topology model of the LH/CG receptor. In some receptors, the flag epitope, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (shown in squares), was inserted at the N terminus of the mature receptor lacking the signal sequence. The conservation of Pros is shown for glycoprotein hormone receptors and other seven-TM receptors. Conserved and diverse residues are marked as “+” and “−”, respectively.

### RESULTS

There are five Pros in the seven TM helices of the LH/CG receptor, Pro463 and Pro479 in TM 4, Pro562 in TM 6, and Pro591 and Pro598 in TM 7 (Fig. 1). These TM Pros were individually substituted with Phe to produce LH/CG-RP463F, LH/CG-RP479F, LH/CG-RP562F, LH/CG-RP591F, and LH/CG-RP598F. The mutant receptor plasmids were transfected into 293 cells. Stably transfected cells were assayed for 125I-hCG binding and hCG-dependent cAMP induction. Counts of empty tubes (background) were ~50 cpm, and nonspecific binding was ~100 cpm, including background. Maximum specific binding counts/min are included in parentheses in the table section of the figure. Intact receptors/cell, EC50 for cAMP induction, and % change from wild type receptor are shown in Table 1. In addition, values for different mutants were compared with the corresponding values of the wild type receptor using analysis of variance with 95% confidence. A mutant with p < 0.05 was considered to be a significantly different from the wild type as discussed in the text.

Radioimmunoassay for Flag-LH/CG Receptors—Mouse anti-flag monoclonal antibody M2 (Eastman Kodak Co.) was iodinated with 125I according to the published procedure for radioiodination of hCG (17), and 125I-anti-flag antibodies were purified on a Sephadex G-150 column. Binding of 125I-anti-flag to 293 cells expressing flag-LH receptors was carried out following the 125I-hCG binding assay described above.

### FIG. 2. Biological activities of Pro → Phe substitution mutants.** Five TM Pros were individually substituted with Phe and the mutant receptors were stably expressed in human 293 cells. The cells were assayed for hormone binding and hCG-dependent cAMP induction. Counts of empty tubes (background) were ~50 cpm, and nonspecific binding was ~100 cpm, including background. Maximum specific binding counts/min are included in parentheses in the table. Each experiment was performed in duplicate, and values were determined for Kd, receptors/cell, EC50 for cAMP synthesis, and maximal cAMP level. Experiments were repeated four to six times, and means and S.D. were presented in the table. The statistical significance of the data was analyzed by Student’s t test. The resulting p values are presented as a p < 0.001, b for p < 0.01, c for p < 0.02, and d for p < 0.05. NS, not significant; ND, not detectable. In addition, values for different mutants were compared with the corresponding values of the wild type receptor using analysis of variance with 95% confidence. A mutant with p < 0.05 was considered to be a significantly different from the wild type as discussed in the text.

| TM | p463 | p479 | p562 | p591 | p598 |
|----|------|------|------|------|------|
| LH/CG-R | + | + | + | + | + |
| FSH-R | + | + | + | + | + |
| TSH-R | + | + | + | + | + |
| 7 TM-R | + | - | + | + | + |

### TABLE 1

| TM | p463 | p479 | p562 | p591 | p598 |
|----|------|------|------|------|------|
| Kd (nM) | 80 ± 5 | 75 ± 13 | 75 (4.69) | 650 ± 179 | 76 ± 13 | (3,300) |
| EC50 (nM) | 1150 ± 175 | 43 ± 6 | (1,070) | 67 ± 20 | 76 ± 9 | d |
| Max (fold) | ND | ND | ND | ND | ND |

### FIG. 3

**Radioimmunoassay for Flag-LH/CG Receptors.**

- **A. Binding to Cells**
- **B. Binding in Solution**
- **C. cAMP Synthesis**

Lys (5’-GAC TAC AAG GAC GAT GAC GAT AAG-3’), was inserted between the C terminus (Ser26) of the signal sequence and the N terminus of the mature receptor (Arg27). The flag epitope (19) has successfully been used as a marker to identify, trace, and purify recombinant proteins carrying the tag without significantly impairing their biological activities (20, 21). Intact cells were cultured on coverslips in 6-well plates for 2 days and fixed with 4% formaldehyde in PBS for 10 min at 25°C. For labeling permeabilized cells, cells were fixed with 4% formaldehyde in PBS for 5 min at 4°C and treated with 0.1% Triton X-100 in PBS for 5 min at 4°C. Fixed intact or permeabilized cells were washed five times with PBS for 2.5 min each at 25°C. They were sequentially treated with 0.4% type IV gelatin isolated from calf skin (Sigma) in modified Eagle’s medium free of phenol red for 10 min at 25°C and then with 5% goat serum and 1% fetal calf serum in the same medium for 20 min at 25°C. The treated cells were incubated with 500 μl/well of primary antibody solution (25 μg of mouse anti-flag antibody in 1 ml of modified Eagle’s medium containing 5% goat serum and 1% fetal calf serum) for 2 h at 37°C. The cells were washed three times with PBS for 2.5 min each at 25°C and treated with a 400-fold dilution of Texas Red conjugated to goat anti-mouse IgG (Molecular Probes). Finally, the cells were washed with PBS six times for 2.5 min each at 25°C. The coverslip containing processed cells was mounted on glass slides using 50% glycerol in PBS and sealed using nail polish. Specimens were examined under a Leica TCS-4D laser scanning confocal microscope equipped with Scanware analysis software. The entire experiments were completed in a day to prevent increasing background fluorescence.
than that of the wild type receptor (Fig. 2C). These results suggest that the high EC\textsubscript{50} values are due to the corresponding high $K_d$ values. Also these hCG binding and hCG-dependent cAMP induction are specific for the receptor since nontransfected cells and mock transfected cells did not bind $^{125}$I-hCG nor induced hCG-dependent cAMP production. In contrast to the functional mutants, LH/CG-RP479F and LH/CG-RP598F did not bind hCG or induce cAMP production at the normal hCG concentrations. However, at $>\mu M$ hCG concentrations, LH/CG-RP479F showed some hCG binding but not cAMP induction (data not shown). This result does not clearly distinguish whether the apparent low affinity hormone binding of LH/CG-RP479F is due to a reduction in binding affinity or to low surface expression.

**Solubilized Receptors**—Since these two mutant receptors might be synthesized and trapped within cells, $^{125}$I-hCG binding studies were performed with cells solubilized in Nonidet P-40. hCG binding to solubilized LH/CG-RP479F was barely detectable. In contrast, solubilized LH/CG-RP598F bound hCG (Fig. 2B) with affinity similar that of solubilized wild type receptor, LH/CG-RP463F, LH/CG-RP562F and LH/CG-RP591F.

However, the $K_d$ values of solubilized receptors are $\sim2$-fold higher than the corresponding receptors on intact cells. This small loss in apparent binding affinities of solubilized receptors is often observed for wild type and mutant receptors (22). It may reflect either the low sensitivity of the assay or slight denaturation of receptors during the solubilization and/or assay steps. In any event, our data indicate that LH/CG-RP598F is synthesized and capable of hCG binding. Furthermore, the result suggests that the mutant receptor might be trapped inside of the cells. Alternatively, LH/CG-RP598F may be expressed on the cell surface but is incapable of binding hCG in situ, perhaps due to defective folding. In this case, solubilization in the nonionic detergent may facilitate proper folding and promote hormone binding. In contrast, LH/CG-RP479F is either not synthesized or synthesized but incapable of binding hormone.

**Antibody Binding**—To further analyze expression and localization of the Pro\textsuperscript{479}\textsuperscript{3}Phe and Pro\textsuperscript{598}\textsuperscript{3}Phe mutants, two independent immunological methods were utilized. Cells were transfected with plasmids encoding receptors carrying the flag epitope, flag wild type LH/CG receptor, flag-LH/CG-RP479F, and flag-LH/CG-RP598F. For immunofluorescent labeling studies, cells were either examined intact or after treatment with Triton X-100 to permeabilize the plasma membrane and to allow the antibody to enter the cytosol. Confocal laser fluorescence microscopy shows bright fluorescence of the flag wild type receptor on intact cells and in permeabilized cells (Fig. 3). Cells expressing the wild type receptor lacking the flag tag did not show fluorescence, regardless of permeabilization. In addition, the cells expressing flag wild type receptor did not show fluorescence when treated for fluorescence labeling without anti-flag antibody. These controls demonstrate that the flag wild type receptor is expressed on the cell surface and within cells. Flag-LH/CG-RP479F was also observed on intact and permeabilized cells, indicating it is expressed on the cell surface and within cells. On the other hand, flag-LH/CG-RP598F was observed in permeabilized cells only, indicating that flag-LH/CG-RP598F was not transported to the cell surface.

The other approach used to test expression of the receptors was radioimmune ligand binding using radioiodinated monoclonal anti-flag antibody. $^{125}$I-anti-flag antibody bound to the cells expressing the flag wild type receptor or flag-LH/CG-RP479F but not to the cells expressing flag-LH/CG-RP598F (Fig. 4). The $K_d$ values were 29 nM and 147 nM for the flag wild type receptor and flag-LH/CG-RP479F, respectively. The number of binding sites on the cell surface are significant, 27,000 and
138,000/cell, excluding the possibility that the binding observed resulted from nonspecific binding. Taken together, these and the fluorescence microscopy indicate that 125I-anti-flag was capable of specifically detecting the flag epitope in the receptors. More importantly, the results demonstrate that the Flag-wild type receptor and flag-LH/CG-RP479F were expressed on the cell surface, whereas flag-LH/CG-RP598F was not.

Activities of Flag Receptors—To test whether the flag epitope might interfere with processing and activities of the flag-LH/CG receptors, hCG binding and cAMP induction were examined. The flag wild type receptor and the wild type receptor on intact cells and in solution bound hCG with similar affinities (Fig. 5, A and B). In addition, the flag wild type receptor was capable of hCG-dependent cAMP induction, although the EC50 value for cAMP induction was ~3-fold higher than the value of the wild type receptor (Fig. 5C). Furthermore, the Kd values of solubilized LH/CG-RP598F and flag-LH/CG-RP598F were similar (Figs. 2B and 5B). These data show that the flag-LH/CG receptors are active, although their potency is somewhat different from LH/CG receptors lacking the flag epitope. With this in mind, we examined flag-LH/CG-RP479F. While hCG did not bind to flag-LH/CG-RP479F on intact cells, hCG bound to the receptor in detergent solution with low affinity (Fig. 5B). This is not entirely surprising since hCG binding to LH/CG-RP479F in solution was barely detectable.

The loss of binding on intact cells and the extremely low affinity binding in solution of flag-LH/CG-RP479F could be due to the introduction of the bulky phenyl side chain or the loss of the Pro induced kink at this position in TM helix 4. To distinguish these possibilities, Pro279 was substituted with Gly and Ala to produce Flag-LH/CG-RP479G and Flag-LH/CG-RP479A, respectively. Both mutants failed to bind hCG on intact cells and induce hCG dependent cAMP synthesis (Fig. 6, A and B). On the other hand, they were capable of binding hCG in detergent solution and induce hCG dependent cAMP synthesis (Fig. 6, A and B). The Kd values were ~2-fold higher than that of the flag wild type receptor. In addition, receptor concentrations were greater than that of the flag wild type receptor. Again, it appeared possible that both mutants may not be expressed on the cell surface or may be expressed on the surface but defective in hCG binding. In the latter case, they may have undergone changes in the structure when solubilized in the detergent solution. To resolve these possibilities, cells transfected with plasmids carrying the mutant cDNAs were treated with anti-flag antibodies and examined under confocal microscopy. The results in Fig. 7 demonstrate that flag-LH/CG-RP479G and Flag-LH/CG-RP479A are present both on the surface of intact cells and within permeabilized cells.

DISCUSSION

In this study, the five TM Pros of the LH/CG receptor were individually substituted with Phe and the resulting mutant receptors were examined for hormone binding and cAMP in-
hormone binding. In contrast, the Pro479 substitution prevented surface expression without affecting solubilization. Which were present in the membrane but removed by detergent might be interfered with due to other membrane molecules. It is also possible that hormone binding of the mutants lipid bilayer or associated with the nonionic detergent in solution. As a simple explanation is that, during solubilization, the mutant receptors undergo structural changes leading to a structure more closely resembles the native receptor. Alternatively, the side chain of Phe479 interferes the interaction of TM 4 with a neighboring TM, hormone binding, and/or structural restoration during solubilization. A similar result was observed when Pro479 of the thyrotropin receptor, which is equivalent to Pro479 of the LH/CG receptor, was replaced with Leu in the hypothyroid hyt/hyt mouse (23, 24). The thyrotropin receptor mutant was expressed on the cell surface but was incapable of binding hormone. Therefore, the substitution of bulky amino acids for the TM 4 Pro appears to be intolerable for hormone binding at the cell surface. Also, the Pro479-induced kink does not appear to play a significant role in hormone binding, since the Pro479 → Gly and Pro479 → Ala substitutions had the same effect, although the helix is expected to bend by Gly but not by Ala. Taken together, these results indicate that the side chain of Pro479 is more important than the Pro479-induced kink for hormone binding, particularly at the cell surface. On the other hand, Pro479 is not required for surface expression of the receptor.

The substitution of Phe for Pro463 of TM 4, Pro562 of TM 6, and Pro591 of TM 7 slightly increased the $K_d$ values for hCG binding on intact cells and in detergent solution as well as the EC$_{50}$ values for cAMP induction. However, the substitutions did not significantly impact the maximal cAMP level. The results indicate that these three TM Pros play marginal roles on hormone binding and cAMP induction, and have no effect on surface expression. The marginal effect of the Pro463 → Phe substitution is not surprising. Pro463 in TM 4 is variant among the LH/CG receptors of different species and is generally absent in other glycoprotein hormone receptors and seven TM receptors. On the other hand, Pro479 of TM 4 and Pro591 of TM 7 are conserved throughout seven TM receptors. Likewise, Pro562 of TM 6 and Pro591 of TM 7 are conserved among the glycoprotein hormone receptors and are partially conserved among other seven TM receptors. Pro463 may be nonessential because it is located at the third amino acid position from the N terminus of the TM 4 helix. Although internal Pros are helix destabilizers, a Pro within the first three positions does not destabilize an α helix (12), because amide nitrogens of the first four residues in an α helix do not form hydrogen bonds.

Pro562 is located in the middle of TM 6 and Pro591 in the N-terminal one-third of TM 7. Consequently, the loss of either Pro463 may affect exoloop 3. Since these Pro → Phe substitutions only slightly reduced the high affinity for hormone binding, exoloop 3 may not play a crucial role in high affinity hormone binding. These findings are consistent with the observation that exoloop 3 of the LH/CG receptor is essential for receptor activation but is only marginally important for high affinity hormone binding$^2$ (22). Also the results are in accord with studies showing that a synthetic peptide corresponding to the exoloop 3 sequence of the LH/CG receptor inhibits $^{125}$I-hCG binding to the LH/CG receptor only slightly (25).

Finally, the data in this study also demonstrate the utility of flag epitopes for the localization, transport, and quantification of glycoprotein hormone receptors. Introduced epitopes do not significantly impact the biological activity of the receptor, which in turn provides a sound methodology for studying their surface expression and activation.

**REFERENCES**

1. McFarland, K., Sprengel, R., Phillips, H., Kohler, M., Rosenthal, N., Nikolos, K., Segaloff, D., and Seeburg, P. (1988) Science 245, 494–499

2. S. Hong, M.-S. Oh, K.-S. Ryu, I. Ji, and T. H. Ji, unpublished observation.
2. Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Thi, M., Jolivet, A., Guiochon-Mantel, A., Sar, S., Jallal, B., Garnier, J., and Milgrom, E. (1989) *Science* **245**, 525–528
3. Tsai-Morris, C. H., Buczko, E., Wang, W., and Dufau, M. L. (1990) *J. Biol. Chem.* **265**, 19385–19388
4. Xie, Y.-B., Wang, H., and Segaloff, D. L. (1990) *J. Biol. Chem.* **265**, 21411–21414
5. Ji, I., and Ji, T. H. (1991) *Endocrinology* **128**, 2648–2650
6. Remy, J. J., Bozon, V., Couture, L., Gene, B., Salesse, R., and Garnier, J. (1993) *Biochem. Biophys. Res. Commun.* **193**, 1023–1030
7. Ji, I., and Ji, T. H. (1991) *J. Biol. Chem.* **266**, 10307–10309
8. Shenker, A., Lauer, L., Kougi, S., Merendino, J., Jr., Minegishi, T., and Cutler, G., Jr. (1990) *Nature* **365**, 652–654
9. Ji, I., and Ji, T. H. (1993) *J. Biol. Chem.* **268**, 20851–20854
10. Huang, J., and Puett, D. (1995) *J. Biol. Chem.* **270**, 30023–30028
11. Probst, W., Snyder, L., Schuster, D., Brosius, J., and Sealfon, S. (1992) *DNA Cell Biol.* **11**, 1–20
12. von Heijne, G. (1991) *J. Mol. Biol.* **218**, 499–503
13. Chou, P., and Fasman, G. (1978) *Adv. Enzymol.* **47**, 45–148
14. Barlow, D., and Thornton, J. (1988) *J. Mol. Biol.* **201**, 601–619
15. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) *Nature* **318**, 618–624
16. Ji, T., Murdoch, W., and Ji, I. (1995) *Endocrine* **3**, 187–194
17. Ji, I., and Ji, T. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 5465–5469
18. Prikett, K., Amberg, D., and Hopp, T. (1989) *Biotechniques* **7**, 580–589
19. Hopp, T., Prikett, K., Price, V., Libby, R., March, C., Cerretti, P., Urdal, D., and Conlon, P. (1988) *Bio/Technology* **6**, 1205–1210
20. Guan, X.-M., Kobilka, T. S., and Kobilka, B. K. (1992) *J. Biol. Chem.* **267**, 21995–21998
21. Chiang, C., and Roeder, R. (1993) *Pept. Res.* **6**, 62–64
22. Ryu, K.-S., Gilchrist, R. L., Ji, I., Kim, S.-J., and Ji, T. H. (1996) *J. Biol. Chem.* **271**, 7301–7304
23. Stein, S. A., Oates, E. L., Hall, C. R., Grumbles, R. M., Fernandez, L. M., Taylor, N. A., Puett, D., and Jin, S. (1994) *Mol. Endocrinol.* **8**, 129–138
24. Gu, W.-X., Du, G.-G., Kopp, P., Rentonius, A., Albanese, C., Kohn, L., Madison, L., and Jameson, J. (1995) *Endocrinology* **136**, 3146–3153
25. Roche, P. C., Ryan, R. J., and McCormick, D. J. (1992) *Endocrinology* **131**, 268–274