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The extracellular domain of luteinizing hormone receptor dictates its efficiency of maturation ☆

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ABSTRACT

The processing of luteinizing hormone receptor (LHR) shows marked differences in different species. While the human LHR is predominantly expressed as the mature, 90 kDa species, rat LHR exists mostly in the 70 kDa precursor form. Since the extracellular domain of the LHR is unusually large in comparison with other G protein-coupled receptors, the present studies examined the role of extracellular domain in its processing. FLAG-tagged chimeric LH receptors were constructed by substituting the extracellular domain of the human receptor in rat LHR (*hrr*) and the extracellular domain of the rat receptor in human LHR (*rhh*). The intracellular processing, ligand binding and recycling of the chimeric receptors were compared with that of the wild type receptors in 293T cells. The results showed that the human and rat LHR were expressed predominantly as 90 and 70 kDa species, respectively, as expected. The introduction of the rat extracellular domain into the human LHR (*rhh*) decreased the abundance of the mature form with an increase in the precursor form. Conversely, substitution of the extracellular domain of the rat LHR by the extracellular domain of the human LHR (*hrr*) led to an increase in the mature form with a corresponding decrease in the precursor form. Changes were also observed in the ligand binding and recycling of the wild type and chimeric receptors. These results suggest that the extracellular domain of the LHR is one of the determinants that confer its ability for proper maturation and cell surface expression.

The luteinizing hormone receptor (LHR) is a glycoprotein hormone receptor that belongs to the rhodopsin/β_2-adrenergic receptor subfamily of G protein-coupled receptors (GPCR) [1,2]. Previous studies have shown that the rat LH receptor exists predominantly in the precursor, immature form, suggesting that the receptor is not efficiently processed into mature, cell surface form [3,4]. The immature rat LHR is thought to be retained in the endoplasmic reticulum where it eventually undergoes degradation. Studies have demonstrated that inefficient processing of the rat LHR is not a result of overexpression or heterologous expression in cell culture as this immature form is also present in primary tissues [5]. A larger proportion of the human LHR, by contrast, exists predominantly as the mature, 90 kDa from [6]. It is uncertain why the maturation efficiency differs between species with over 85% homology in their amino acid sequence. It is likely that differences in the amino acid sequence of rat and human LHR may lead to the different efficiencies of processing.

Since the extracellular domain of the LHR is unusually large, it is likely that folding of this domain might be an important step in receptor maturation. In the present study, chimeras were constructed to test if the extracellular domain dictates the processing efficiency of the LHR. Previous studies have shown that the human LHR is recycled back to the cell surface more efficiently than the rat LHR. Therefore, the recycling of the chimeric receptors was examined to determine whether the extracellular domain of the LHR plays any role in recycling. Our results suggest that the structure of the extracellular domain of the LHR plays an important role in the intracellular processing of the receptor.

Materials and methods

Materials. Human embryonic kidney cells (293T cells) expressing the large T antigen were a gift from Dr. G.P. Nolan, Stanford University, California. Highly purified human chorionic gonadotropin (CR-127) was purchased from Dr. Al Parlow, UCLA. Chloramine T and anti-FLAG antibodies were purchased from Sigma (St. Louis, MO). Fugene transfection reagent was from Roche (Indianapolis, IN). Waymouth's MB752/1 medium, DMEM (Dulbecco's modified Eagle's medium), Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were purchased from GIBCO (Carlsbad, CA). Protease inhibitors were from Boehringer Mannheim (Indianapolis, IN). Calnexin antibody was from StressGen (Victoria, BC, Canada). All other reagents used were in a suitably purified form.

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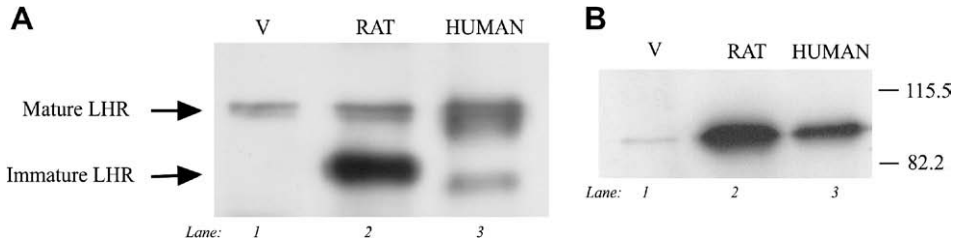

Fig. 1. Expression of wild type human LHR and rat LHR in transfected cells. Transiently transfected 293T cells expressing the rat and human LHR were solubilized, precleared, immunoprecipitated with FLAG M2 antibody and analyzed by Western blot analysis using (A) LHR antibody or (B) calnexin antibody. Lane 1 represents cells that were mock-transfected with plasmid alone. Lanes 2 and 3 represent transfections with rat and human LHR, respectively.

Construction of chimeric receptors. To construct the *rhh* chimeric receptor, the extracellular domain of the rat luteinizing hormone receptor was PCR-cloned using a 5′ primer that contained a *NotI* restriction site for ligation into the vector pFLAG-CMVI and a 3′ primer that contained sequences spanning the 3′ end of the rat extracellular domain and the 5′ end of the human LHR transmembrane domain. The transmembrane domain and the cytoplasmic tail of the human LHR were PCR-cloned using a 5′ primer that contained sequences from the 3′ end of the rat LHR extracellular domain and a 3′ primer that contained a *BamHI* restriction site for ligation into the vector. The rat LHR extracellular domain and the human LHR transmembrane domain and cytoplasmic tail were then PCR-cloned using the 5′ primer that contained a *NotI* restriction site and the 3′ primer that contained a *BamHI* restriction site. This fragment and the vector were then digested and then ligated together. The *hrr* chimeric receptor was constructed similarly by ligating the extracellular domain from the human LHR and the transmembrane domain and cytoplasmic tail of the rat LHR.

Cell culture and transient expression of LH receptor. 293T cells were maintained at 37 °C with 5% CO_2 in DMEM containing 10% fetal bovine serum, 9 U/ml nystatin and 50 μg/ml gentamycin. For transfections, cells were plated 6–8 h before transfection at a density of 2.5–4 × 10^6 cells/10 cm dish, 1.5–2.5 × 10^6 cells/6 cm dish or 3.5 × 10^5 cells/well in 6-well plates. The DNA concentrations used for transfections were 7 μg/10 cm plate, 4.2 μg/6 cm plate and 0.3–0.5 μg/well in 6-well plates.

Immunoprecipitation and Western blot analysis of the LH receptor. Immunoprecipitation of the LH receptor was performed as previously described [7].

Binding of [^{125}I]-hCG to wild type and chimeric LH receptors. Highly purified hCG (CR-127) was radio-iodinated using the previously described chloramine T procedure [8]. Binding of hCG to intact transfected 293T cells was performed by incubating with [^{125}I]-hCG for 20 h at 4 °C in a volume of 0.3 ml as previously described [9]. Non-specific binding was determined by including a 1000-fold excess of unlabeled hCG, and specific binding was calculated by subtracting non-specific binding from total binding. The cell surface binding was normalized on the basis of the total number of cells used for the binding assays.

Recycling of internalized hormone. Cells expressing the wild type or chimeric receptors were preincubated in 35 mm wells in assay medium for 30–60 min at 37 °C followed by incubation with 35 ng/ml [^{125}I]-hCG (with 35 μg of unlabeled hCG for designated non-specific binding) for 2 h at 37 °C to allow internalization to occur. Recycling of the internalized [^{125}I]-hCG bound receptor was determined as previously described [6,10].

Assay of cyclic AMP. Cells expressing the WT and chimeric receptors were incubated in 35 mm wells with 0.5 mM 3-isobutyl-1-methylxanthine in Assay Medium at 37 °C for 15 min followed by incubation with 0 or 100 ng/ml hCG at 37 °C for 30 min. Media were then removed and cells were harvested with PBS-EDTA. cAMP was assayed using cyclic AMP Assay Kit (GE Healthcare Bio-Sciences Corp. Piscataway, NJ) as previously described [11].

Statistical analysis. SigmaStat was used to perform One-way ANOVA analysis to determine statistical significance with $P < 0.05$ considered significant.

Results

To examine if rat and human LHR are processed with different efficiencies, the expression of rat and human LHR was examined by Western blot analysis. The results in Fig. 1A show that the rat LHR is primarily expressed as the low molecular weight form (approximately 70 kDa), which has previously been identified as the intracellular, precursor form of the receptor [3,12]. Conversely, the human LHR appears as a 90 kDa form representing the fully processed receptor along with a less intense band detected as the 70 kDa precursor. These results show that the human LHR exists predominantly in the mature form while the rat LHR exists primarily in the precursor form when expressed in 293T cells, suggesting that the human LHR is processed more efficiently than the rat LHR.

Previous studies have shown that the immature forms of the glycoprotein hormone receptors, including LH receptor, interact with the chaperone calnexin in the endoplasmic reticulum [13]. To confirm that a greater portion of the rat LHR exists in the precursor form associated with the endoplasmic reticulum, the extent of association of the rat and human LHR with calnexin was compared by immunoprecipitating the receptors followed by Western blot analysis using calnexin antibody. As shown in Fig. 1B, calnexin associates with rat LHR (lane 2) to a greater extent than the human LHR (lane 3). This finding further supports the observation that rat LHR is predominantly expressed as the intracellular, immature form and human LHR is predominantly expressed in its mature form. The identity of these two forms has been extensively characterized in previous studies [3,12].

The extracellular domain of the LHR is larger than most other G protein-coupled receptors [1]. Thus, to examine if the differences in the extracellular domain might be responsible for the different efficiencies with which the receptor is processed, chimeric receptors were constructed. *rrr* and *hhh* represent the full-length rat and human LHR, respectively. The *hrr* chimera contained the extracellular domain of the human LHR and the transmembrane and carboxy terminal of the rat LHR while *rhh* chimera contained the extracellular domain of the rat LHR and the transmembrane and carboxy terminus of the human LHR. To test if the extracellular domain dictates LHR processing efficiency, all constructs (*hhh*, *rrr*, *rhh*, or *hrr*) expressed in 293T cells were lysed, LHR immunoprecipitated with anti-FLAG antibody and subjected to Western blot analysis. The different molecular forms of the receptor detected after transfections with these constructs are shown in Fig. 2. The mock-transfected control, in lane 1, showed no bands, as expected. Lane 4 shows expression of the wild type rat LHR (*rrr*). A predominant band was detected at approximately 70 kDa, indicating that the receptor is expressed primarily as the precursor form. Lane 2 shows expression of the *rhh* chimeric receptor. Substitution of

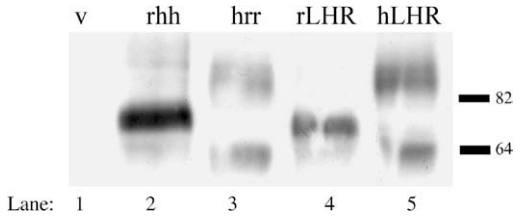

Fig. 2. Expression of chimeric LHR. Transiently transfected 293T cells expressing chimeric rat and human LHR were immunoprecipitated with FLAG M2 antibody and analyzed by Western blot. The vector alone (lane 1) was used as a negative control. Lane 2 represents *rhh* chimeric receptor and lane 3, the *hrr* chimeric receptor. Lanes 4 and 5 represent rat and human LHR, respectively.

the extracellular domain of the human LHR with the extracellular domain of the rat LHR led to the formation of the precursor form rather than the mature form. On the other hand, the *hrr* chimera and native human LHR (*hhh*), showed a strong band at approximately 90 kDa (lanes 3 and 5, respectively), indicating that they both exist predominantly in the mature, fully processed form.

To confirm that the predominance of the 90 kDa form of the receptor correlates with increased receptor expression at the cell surface, binding analysis was performed as described in Material and methods. The percentages of hCG binding by the chimeric and native receptors are represented in Fig. 3. The human (*hhh*) receptor is used as the reference point and is set at 100% because it consistently showed the highest level of ligand binding compared to the other three constructs. Results presented in Fig. 3 show that the *hrr* chimera demonstrated significantly higher binding compared to the native rat (*rrr*) receptor, whereas the *rhh* chimera showed less binding compared to the human (*hhh*) receptor. Thus, receptors with rat extracellular domain (*rrr* and *rhh*) exhibited a tendency for lower hCG binding than those with human extracellular domain (*hhh* and *hrr*). Since the transfection efficiencies of different LHR constructs might vary, it is not possible to establish a direct correlation between the prevalence of the 90 kDa form and ligand binding ability on a quantitative basis. However, there is a trend between an increase in [^{125}I]-hCG binding and the prevalence of the mature form of the receptor.

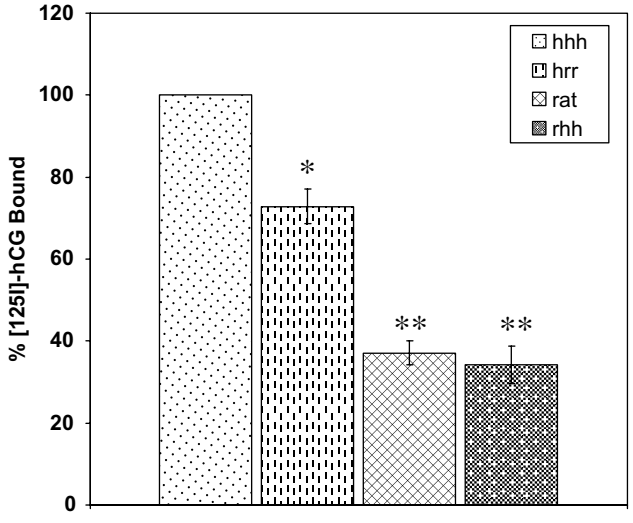

Fig. 3. Cell surface [^{125}I]-hCG binding of chimeric LHR. Binding of [^{125}I]-hCG to intact transfected 293T cells was performed using a single high concentration of [^{125}I]-hCG in the presence or absence of a 1000-fold excess of unlabeled hCG. Non-specific binding was subtracted from the total binding to obtain specific binding. The amount of hCG bound to the *hhh* receptor was used as a reference point and set at 100%. Statistical analysis was done using One-way ANOVA. $^{*}P<0.01$ *hrr* vs *hhh*, $^{**}P<0.001$ *rhh* vs *hhh* and rat vs *hhh*.

Table 1
Cyclic AMP production by cells expressing WT and chimeric LH/hCG receptors.

hCG	cAMP (pmol/μg protein)	
	(−)	(+)
rrr	0.01 ± 0.001	76.11 ± 1.37
hrr	0.02 ± 0.002	94.32 ± 1.95
rhh	0.04 ± 0.002	69.43 ± 3.92
hhh	0.02 ± 0.002	69.17 ± 4.97

293T cells transiently transfected with WT rat (*rrr*), human (*hhh*) or chimera (*hrr* or *rhh*) LH/hCG receptor cDNA were incubated without or with hCG (100 ng/ml) for 30 min. Cyclic AMP was assayed by radioimmuno assay [11].

The responsiveness of the cells expressing different LHR constructs was then tested by exposing these cells to hCG followed by cyclic AMP measurements. The results show that while the hCG binding was much higher in cells expressing the wild type human LHR in comparison to the wild type rat LHR, both were equally effective in cyclic AMP production in response to maximal concentration of hCG. The chimeric receptors, *hrr* and *rhh* were also capable of stimulating cAMP production in response to hCG without any significant change in their responsiveness (Table 1). This is consistent with the notion that not all of the cell surface LHR is functionally coupled to the G proteins [14,15].

The effect of exchanging the extracellular domains between rat and human LHR on recycling was then examined, since human LHR is recycled more efficiently than the rat LHR. The results (Fig. 4A) show that substitution of human extracellular domain in the rat receptor increased its recycling significantly, although it did not reach the levels seen with the wild type human LHR. Conversely, substitution of the extracellular domain of the human LHR with the extracellular domain of the rat LHR reduced its recycling efficiency compared to that of the wild type human LHR (Fig. 4B). Taken together, our data suggest that the extracellular domains of human and rat LHR determine their efficiency of maturation, ligand binding and the extent of recycling.

Discussion

Previous studies have shown that less than 15% of the synthesized rat LHR is transported to the cell surface [3,4] compared to the 80% for the human LHR [2,6]. Inefficient maturation of the rat LHR appears to be an inherent property of this receptor, as an abundance of immature receptor is observed not only in cell culture, but also in primary tissues [5].

The LHR has an unusually large extracellular domain that may have an effect on maturation. Thus, this study examined the role of the extracellular domain in determining the efficiency with which the LH receptors undergo processing to the mature, cell surface form. It was seen that the chimeric receptor, *rhh*, displayed a band of high intensity at approximately 70 kDa, corresponding to the molecular weight of the immature receptor. This suggests that the efficiency of the conversion of precursor to mature form of the human LHR is impaired by the presence of the extracellular domain of the rat LHR (see Fig. 2, lanes 2 and 4). By contrast, the chimeric receptor *hrr* displayed a prominent band at approximately 90 kDa suggesting that LHR containing the human extracellular domain is expressed predominantly as the mature form (Fig. 2, lanes 3 and 5).

Binding as well as recycling analysis also support the hypothesis that the extracellular domain of the LHR dictates processing efficiency (Figs. 3 and 4) suggesting that it is the extracellular domain of the rat LHR which retards the receptor from reaching the cell surface. This is the first study that shows the importance of the extracellular domain in determining the efficiency of maturation and recycling of LH receptors. Previous studies have shown a role

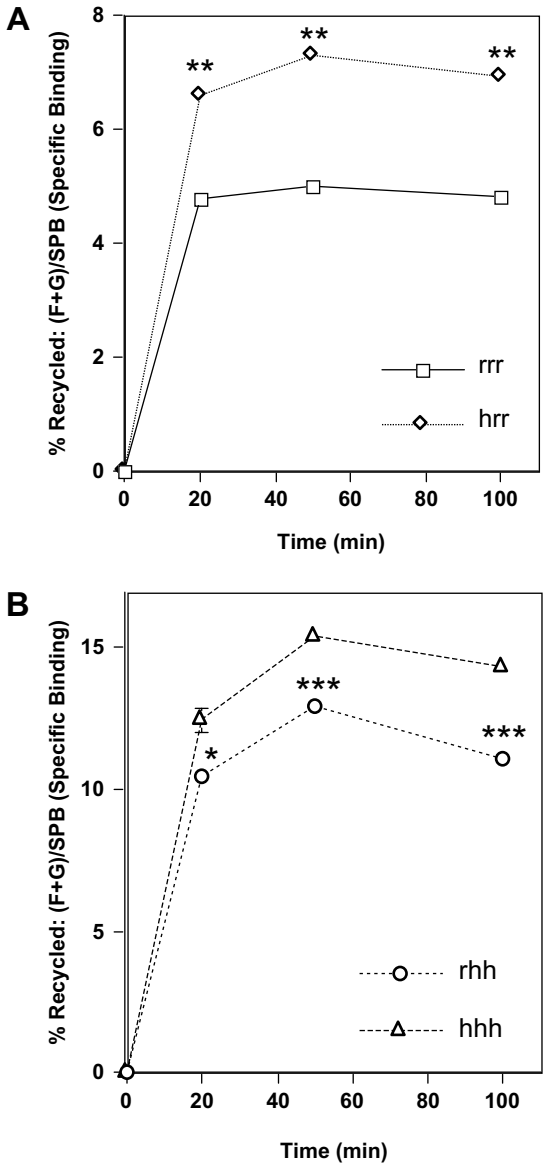

Fig. 4. Recycling of the wild type and chimeric LHR constructs. Transiently transfected 293T cells were assayed for receptor recycling as previously described [6,10]. Results are expressed as % of the total specific radioactivity present at $t = 0$. (A) Comparison of recycling: WT rat receptor (*rrr*) vs chimeric rat receptor with human extracellular domain (*hrr*). (B) Comparison of recycling: WT human receptor (*hhh*) vs chimeric human receptor with rat extracellular domain (*rhh*). Each point represents the mean ± SEM of three independent transfections. $^{**}P < 0.005$ *hrr* vs *rrr*, $^{***}P < 0.005$ *rhh* vs *hhh*, $^{*}P < 0.05$ *rhh* vs *hhh*.

of the carboxy terminal tail in determining the recycling efficiency of rat or human LH receptor. The GT structural motif present in the C-terminal tail of human LH receptor when grafted into the rat LH receptor redirected most of the internalized rat LH receptor to a recycling pathway [16]. The above study also showed that while replacing the serpentine and C-terminal domain of the rat LH receptor with that of the human LH receptor increased receptor recycling and decreased the degradation of rat LH receptor, the opposite effect was not observed in human receptor with a complementary manipulation. Thus, it seems likely that the carboxy terminal tail of the rat LH receptor, unlike the human receptor, does not contain sufficient structural information that determines its processing.

Our study implicates that the differences in the processing efficiency between the rat LHR and the human LHR might be due to the changes in the structure of the extracellular domain. For example, the human LH receptor may interact more efficiently with chaperones that assist in receptor folding, thus expediting exit from the ER and maturation. In fact, both the rat and human LHR have been shown to associate with chaperones in the endoplasmic reticulum [13,17]. The rat LHR may interact less efficiently with chaperones, leading to longer intracellular retention possibly leading to inefficient maturation. The amino acid sequences of the human LHR might be more amenable for interaction with chaperones that facilitate proper folding and transport to the cell surface. In support of this notion, there are several studies that suggest that export from ER is the limiting step in the expression of GPCRs and provides a means to regulate the number of functional receptors on the cell surface [18,19]. Recently, it has been shown that enzymatically active SERCA2b interacts with newly synthesized, incompletely folded GPCRs, including rat LHR, in the ER, and that this association has a facilitative effect on receptor maturation and ER export [20]. Interestingly, Pietila et al. showed that proteasomal blockade led to enhanced maturation of the rat LH receptor [5] suggesting that the efficiency of recycling and LHR maturation is tightly coupled to proteasomal function.

The findings in this study may have implications in the understanding of processing in other glycoprotein hormone receptors. Unlike other cell membrane proteins, the regulatory pathways of GPCRs folding and maturation are not widely known. Besides rat LHR, there are other GPCRs that are characterized by inefficient maturation. For example, 50% of the δ opioid receptor is retained in the endoplasmic reticulum and eventually undergoes degradation. Studies by Galet et al. [16] have already shown that addition of portions of C-terminal tail of hLHR to other GPCRs such as the δ opioid receptor promotes their post-endocytotic recycling [16]. Furthermore, from a physiological perspective, there are many known naturally occurring human LHR mutants that display similar inefficient processing and maturation [17,21,22]. The inefficient processing of the rat LH receptor compared to the human LH receptor might be due to the relatively short duration of the estrous cycle in the rat which necessitates rapid changes in the LH receptor expression level by regulating its processing. Although it is still uncertain why the rat LHR is inefficiently processed, the findings in this study demonstrate that the extracellular domain of the LHR dictates its processing efficiency.

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