Biochemical and Biophysical Research Communications 377 (2008) 307–311

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 25 September 2008
Available online 9 October 2008

Keywords:
LH receptor
Extracellular domain
Recycling of receptor
Binding ability

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.

☆ Supported by NIH Grant HD06656.
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doi:10.1016/j.bbrc.2008.09.136

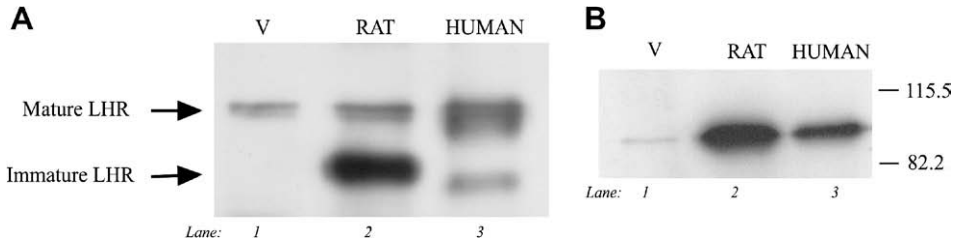

Fig. 1. Expression of wild type human LHR and rat LHR in transfected cells. Transiently transfected 293T cells expressing the rat and human LHR were solubilized, precleared, immunoprecipitated with FLAG M2 antibody and analyzed by Western blot analysis using (A) LHR antibody or (B) calnexin antibody. 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