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Identification and sequence analysis of a second form of prolactin receptor by molecular cloning of complementary DNA from rabbit mammary gland

(peptide hormone receptors/transmembrane signaling/growth hormone/transfection)

MARC EDERY*, CHRISTINE JOLICOEUR†, CORINNE LEVI-MEYRUEIS*, ISABELLE DUSANTER-FOURT*, BARBARA PÉTRIDOU*, JEAN-MARIE BOUTIN†, LAURENCE LESUEUR*, PAUL A. KELLY†, AND JEAN DJIANE*

*Unité d'Endocrinologie Moléculaire, Institut National de la Recherche Agronomique, 78350 Jouy-en-Josas, France; and †Laboratory of Molecular Endocrinology, McGill University, Royal Victoria Hospital, Montréal, PQ, H3A 1A1 Canada

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ABSTRACT **Two λgt11 clones containing fragments of cDNA encoding the prolactin receptor from rabbit mammary gland were isolated using a rat liver prolactin receptor cDNA probe. An 1848-base-pair open reading frame encodes a mature prolactin-binding protein of 592 amino acids that contains three domains: (*i*) the extracellular, amino-terminal, prolactin-binding region of 210 residues; (*ii*) the transmembrane region of 24 residues; and (*iii*) the intracellular, carboxyl-terminal domain of 358 residues. This latter domain is much longer than the cytoplasmic domain (57 residues) previously described for the rat liver prolactin receptor. In addition, the sequence identity of this form of prolactin receptor with the growth hormone receptor is extended in the cytoplasmic domain.**

The anterior pituitary hormone prolactin (PRL) is involved in an impressive list of biological actions in all vertebrates (1) and specific receptors for this hormone have been identified in numerous organs (2). PRL is structurally related to growth hormone (GH) and chorionic somatomammotropic or placental lactogen, which are encoded by the same gene family (3). In mammals, PRL is primarily responsible for the development of the mammary gland and lactation. It stimulates the expression of milk protein genes by increasing both gene transcription and mRNA half-life (4). These actions are initiated by an interaction of PRL with specific, high-affinity receptors located in cell membranes. This receptor has been partially purified from rabbit mammary gland (5) and monoclonal antibodies against it have been developed (6). After binding of PRL to its receptors, the mechanism(s) by which the hormonal signal is transferred inside the cell remains unknown. We have shown (7) that one monoclonal antibody against the PRL receptor is a potent agonist of PRL actions in mammary cells, suggesting that PRL itself is probably not necessary beyond its binding and that the receptor is able, under certain experimental conditions, to operate alone probably by coupling to a second messenger system. However, as is true for GH, none of the classical second messengers (cAMP, cGMP, inositol phospholipids, or calcium ions) appears to be involved in PRL signal transduction (8). The knowledge of the primary structure of the receptor and the identification of functional domains may facilitate a better understanding of these phenomena. To that end, we have previously cloned cDNAs of PRL receptor from rat liver cDNA libraries (9), deduced primary structure of the protein, and established that this receptor has regions of strong sequence homology with the GH receptor, also cloned (10), but contains a much shorter cytoplasmic domain. The mammary gland is a major target organ for PRL, where in contrast to the liver, biological effects are well established (4). We have reported (9) that the major mRNA species for the PRL receptor is much larger in the mammary gland [4 kilobases (kb)] than in the liver (2.2 kb), suggesting heterogeneity in the coding sequence or in the untranslated region of the PRL receptor. To test the possibility that a structurally distinct PRL receptor might exist in the mammary gland, we screened cDNA libraries prepared from rabbit mammary gland. We isolated several cDNA clones and report here that an open reading frame encodes a much larger PRL-binding protein than that in the rat liver. This protein is highly similar to rat liver receptor in the extracellular domain but possesses a much longer cytoplasmic domain (358 amino acids). In addition, the extended cytoplasmic domain has several regions of high similarity with the GH receptor.‡

MATERIALS AND METHODS

Preparation and Screening of cDNA Libraries. The oligo(dT) (1.2×10^6 recombinants) and random-primed (700,000 recombinants) cDNA libraries were constructed from poly(A)$^+$ mRNA prepared from mammary glands of 14-day-pregnant rabbits as follows. Double-stranded cDNA was prepared by published methods (11) and methylated with *Eco*RI methylase. *Eco*RI linkers were ligated to the cDNA and digested with *Eco*RI. Linked cDNAs were purified on Bio-Gel P-60 and ligated to *Eco*RI- and alkaline phosphatase-treated λgt11 arms. Ligation mixtures were packaged with Gigapack (Stratagene) and plated to infect *Escherichia coli* Y1090 (hsdR$^-$, hsdM$^+$). The initial rabbit PRL receptor clone PRL-R$_2$1 (4 kb) was isolated from the oligo(dT)-primed library by screening with the 1.6-kb fragment representing the entire cDNA (1653 nucleotides) from the rat clone F3 (9) labeled by random priming. The hybridization was performed in 20% (vol/vol) formamide at 42°C and the filters were washed in 1× SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7) at 42°C. To isolate clones containing the 5′ end of the receptor, 600,000 clones from the random-primed library were screened with a 5′, 668-base-pair, *Sac* I–*Sac* I (nucleotides 758–1426, Fig. 1) restriction fragment from PRL-R$_2$1. The hybridization was in 50% formamide at 42°C and the filters were washed in 0.2× SSC at 60°C.

DNA Sequencing. Suitable restriction fragments of the cDNA clones were subcloned into M13mp18 and M13mp19 derivatives and both strands were sequenced by the dideoxy-

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Abbreviations: PRL, prolactin; GH, growth hormone.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04510).

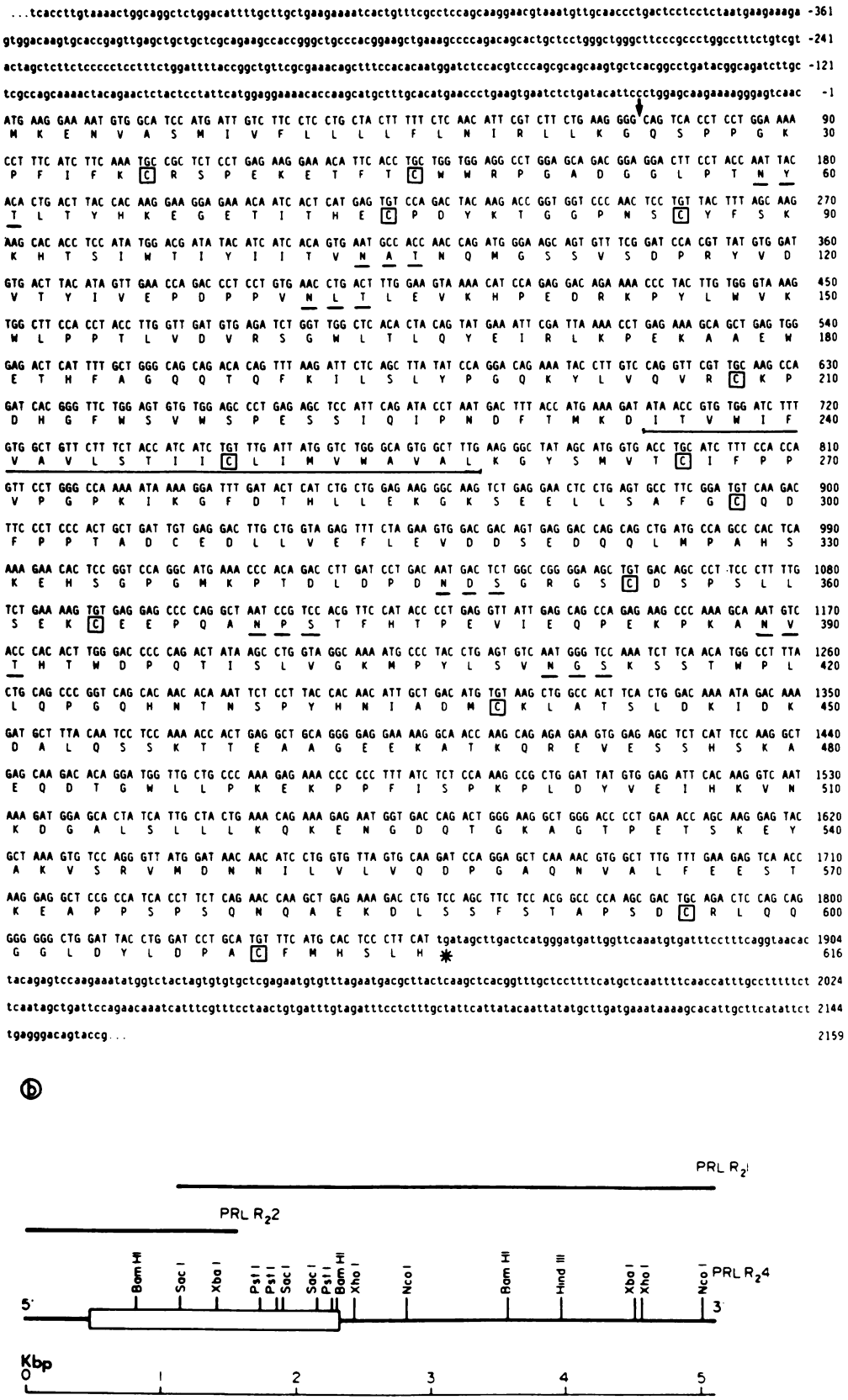

FIG. 1. Maps of the cloned cDNA, nucleotide sequence, and predicted amino acid sequence. (*a*) Nucleotides and amino acids (single-letter code) are numbered at the right in the 5′ to 3′ direction; nucleotide 1 is the adenosine of the initiator ATG codon; negative numbers refer to the 5′ untranslated region. Indicated are the potential cleavage site of the signal peptide (arrow), the putative transmembrane sequence (underlined with the solid line), cysteine residues (boxed), Asn-Xaa-Ser/Thr (NXS/T) sequences for potential N-linked glycosylations (underlined with a dashed line), and the in-frame stop codon (asterisk). The nucleotide sequence presented is shorter than the full-length cDNA clone and thus lacks the cognate 3′ poly(A) sequence. (*b*) Cloned cDNAs. kbp, Kilobase pair(s).

nucleotide chain-termination method using modified T7 DNA polymerase (Sequenase; United States Biochemical).

Expression of the Rabbit PRL Receptor cDNA Clone. The constructed 4.5-kb cDNA containing the full PRL receptor coding sequence was assembled into the mammalian expression vector pECE (12). The cDNA was cloned into *Eco*RI and *Xba* I sites of pECE and was thus placed under the transcriptional control of simian virus 40 early promoter. Transient expression assays were conducted in COS-7 monkey kidney cells. Fifty percent confluent cells were transfected

with plasmid DNA (8 μg) using the calcium phosphate precipitation procedure (13) with the addition of a glycerol shock after 4 hr. After 3 days of culture in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal bovine serum, cells were washed twice with 50 mM Hepes, pH 7.4/150 mM NaCl, scraped with 1 ml of cold 25 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/2 mM EDTA, and lysed in Eppendorf tubes by repeated freeze–thaw cycles. Membranes were prepared by centrifugation (10,000 × *g*) for 5 min and the pellet was resuspended in 25 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. One hundred micrograms of a COS-7 cell membrane suspension was incubated 16 hr at room temperature in the presence of 40,000 cpm of ^{125}I-labeled ovine PRL (≈50 μCi/μg; 1 Ci = 37 GBq) plus various concentrations of unlabeled ligand or other lactogenic or nonlactogenic hormones (see Fig. 3). Binding studies and Scatchard analysis were performed as described (14). Ovine PRL (NIADDK ovine PRL-16; 30.5 international units/mg) and ovine GH (NIADDK ovine GH S11) were kindly supplied by the National Hormone and Pituitary Program (Bethesda, MD). Bovine insulin was obtained from Sigma. Monoclonal anti-receptor antibody M110 was prepared as described (6).

RESULTS

Cloning and Sequencing of Receptor cDNA. The 1.6-kb fragment of the rat liver PRL receptor cDNA clone F3 (9) was used as a probe to screen two cDNA libraries constructed using mRNA prepared from mammary glands from a 14-day-pregnant rabbit. Fig. 1*b* shows a schematic representation of two of the clones (PRL-$R_2$1 and PRL-$R_2$2). The two clones overlap and form an insert of approximately 5 kb. Several species of mRNA were detected in mammary gland using the rabbit cDNA probe in Northern blot analysis (2.8, 4, 6.5, and 10 kb). But, in contrast to what has been shown in rat (9), transcripts of these sizes were found in all rabbit tissues including the liver (data not shown).

Fig. 1*a* shows the sequence of 2635 nucleotides of PRL-$R_2$4, which is a construction made by the two overlapping clones shown in Fig. 1*b*. The open reading frame encodes a 616-amino acid sequence. The first 24 amino acids of the sequence have the characteristics of a classical signal sequence (15). In addition, the sequence preceding the putative initiator ATG codon (AACATG) is consistent with criteria of a translation initiation site (16). The mature form of the encoded protein would thus have 592 amino acids with a theoretical molecular weight of 66,000 and an isoelectric point of 5.66. As shown in Fig. 2, the protein can be divided into three domains: (*i*) the extracellular amino-terminal PRL-binding region of 210 residues that contains five cysteine residues and three consensus sequences for asparagine-linked N-glycosylation; (*ii*) the single transmembrane region of 24 hydrophobic residues, centrally located in the molecule as deduced from the hydropathy analysis (17); and (*iii*) the intracellular, carboxyl-terminal domain of 358 residues containing eight cysteine residues and four potential asparagine-linked N-glycosylation sites.

Expression of Receptor cDNA in Mammalian Cells. The fact that the cDNA clones we selected encoded a protein able to specifically bind PRL was demonstrated by expression in mammalian cells. Transient transfection of the plasmid containing the entire coding region (pECE-PRL-$R_2$3) in COS-7 monkey kidney cells induced the expression of a membrane-bound PRL receptor. Binding experiments with ^{125}I-labeled ovine PRL show that the expressed receptor has a high affinity ($K = 3.5 \times 10^9\ M^{-1}$), similar to that of the microsomal mammary receptor in lactating rabbits ($K = 3 \times 10^9\ M^{-1}$) (14). As expected for the rabbit PRL receptor, known lactogenic hormones (bovine PRL, human GH, or ovine placental lactogen) are able to compete with ^{125}I-labeled

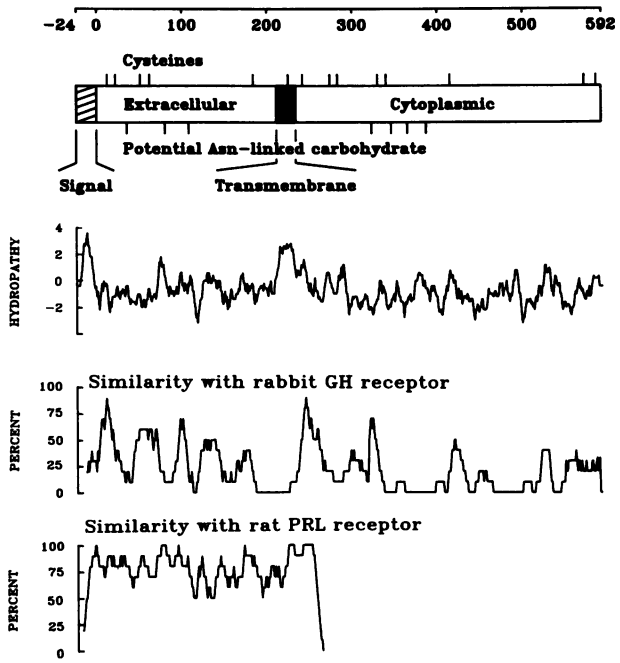

FIG. 2. Schematic representation of the rabbit PRL receptor structure. The hydropathy profile was calculated (17) with a window of 9 residues; positive values indicate increasing hydrophobicity. The potential N-linked glycosylation sites are Asn-Xaa-Ser/Thr (NXS/T). The percentage similarities with the rat PRL receptor and rabbit GH receptor were calculated for exact matches over a window of 10 amino acids.

ovine PRL in the same range of concentrations as unlabeled ovine PRL. No displacement of ^{125}I-labeled ovine PRL was observed with an excess (2 μg/ml) of ovine GH or bovine insulin. A competition assay for PRL binding was also performed in the presence of the anti-rabbit PRL receptor monoclonal antibody M110 (6). As shown in Fig. 3, this antibody totally displaces ^{125}I-labeled ovine PRL from the expressed receptor. These results closely parallel those obtained using rabbit mammary microsomes (6).

Comparison with Rat Liver PRL Receptor and Rabbit GH Receptor Sequences. Computer-assisted searches of several data bases revealed no significant homology between the rabbit PRL receptors and any other reported protein, except PRL and GH receptors (9, 10). As illustrated in Fig. 2, the rabbit and rat PRL receptor protein sequences are highly similar, with 77% overall amino acid identity and 86% overall homology (based on conserved substitutions), when the last 329 amino acids of the cytoplasmic domain of the rabbit receptor are excluded (301 amino acids are absent in the rat liver PRL receptor). All cysteine residues and the three potential extracellular glycosylation sites included in this part of the sequence are conserved in both receptors. There is 33% overall sequence identity and 49% overall similarity (based on conserved substitutions) between rabbit PRL and rabbit GH receptors. Similarity extends beyond the transmembrane region and includes most of the intracellular domain. Fig. 4 shows a schematic representation of the three receptors (rabbit GH and rabbit and rat PRL). Several common regions of striking similarity and additional high-homologous regions between the rabbit PRL and rabbit GH receptor were identified. The highest homology is found in the extracellular regions flanking the first four cysteine residues conserved in the three sequences. Interestingly, the second highest degree of sequence identity (70%) between the three receptors is found in the intracellular region flanking the transmembrane domain. This region is followed by other sequences of moderate similarity (67% and 45%) between the

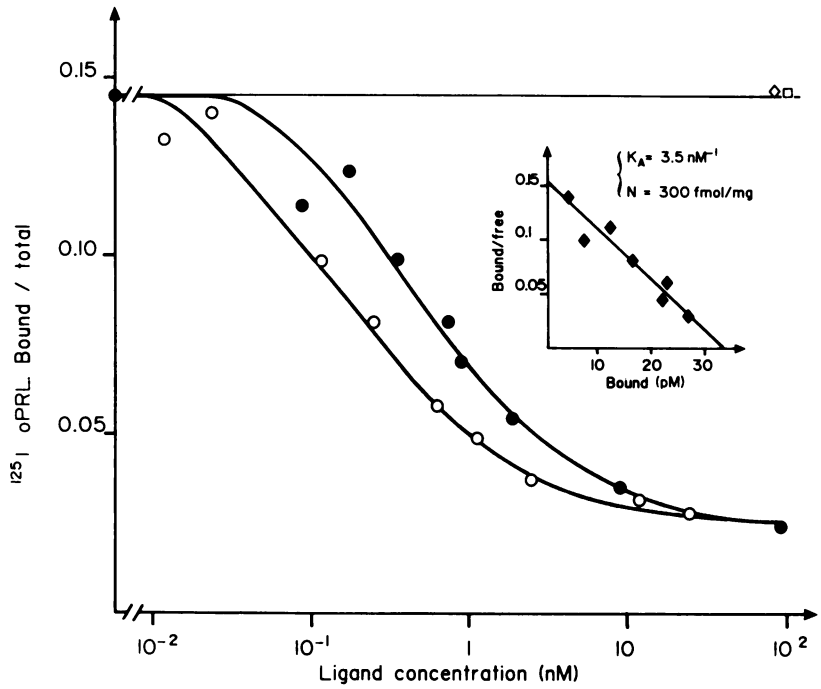

FIG. 3. Expression of the rabbit PRL receptor cDNA clone PRL-$R_2$3 in COS-7 monkey kidney cells. Binding of ^{125}I-labeled ovine PRL (oPRL) to membranes from COS-7 cells transfected with pECE-PRL-$R_2$3 cDNA (Fig. 1). Binding of labeled PRL was studied in the presence of unlabeled PRL (●), anti-PRL receptor monoclonal antibody M110 (○), ovine GH (□), or bovine insulin (◇). No specific binding was observed in untransfected COS-7 cells. The results are expressed as the percent of total cpm bound per 100 μg of protein. (*Inset*) Scatchard plot of the competition assay with unlabeled PRL.

rabbit PRL and GH receptors, in the portion of the sequence not found in the rat liver PRL receptor.

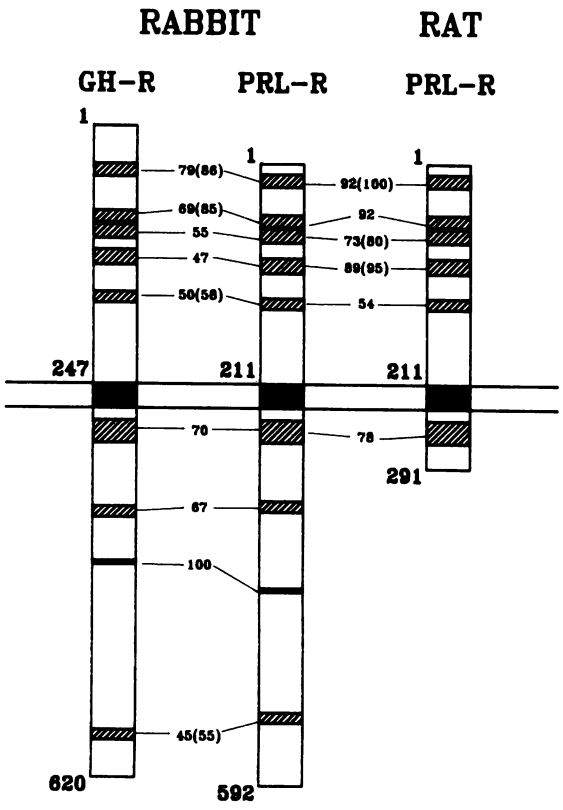

FIG. 4. Schematic representation of the common regions of sequence identity and homology between rabbit PRL, rabbit GH, and rat PRL receptor (R) structures. The three proteins are drawn to the same scale, aligned by their transmembrane (solid boxes) in the plasma membrane with carboxyl termini at the bottom. Structures are based on published primary sequences (9, 10). Numbers indicate the putative position of the first amino acid of the mature protein, the first amino acid of the transmembrane region, and the last amino acid. Common regions of high similarity are indicated by hatched boxes and the percentages of identity and homology (in parentheses) between two receptors are indicated between solid lines.

DISCUSSION

In the present study, we have identified cDNAs in rabbit mammary gland libraries that encode a PRL receptor that differs from the PRL receptor in rat liver. The mammary gland PRL receptor is characterized by a longer cytoplasmic domain that has several regions of high similarity with GH receptor. Thus, for the same peptide hormone, two types of membrane receptors differing in their cytoplasmic domain have been identified and both have been shown to bind the same ligand with equal affinity. This is different from the truncated form of the GH receptor, which is a soluble binding protein lacking the transmembrane region (10), and the v-erbB protein, which is a form of the epidermal growth factor receptor without the growth factor-binding domain (18).

The predicted molecular weight of the rabbit PRL-binding protein (66,000 without glycosylation) is much larger than expected, based on previous studies using cross-linking experiments (19) or immunoprecipitation (20), which indicated that the major form of PRL receptor in several species consists of a single binding unit of M_r ≈40,000. However, a protein of M_r ≈80,000 has been identified in rat ovary (21) and rabbit mammary gland (22). Recent improvements in immunoblotting techniques have permitted the identification of a M_r 77,000 form of the PRL receptor in partially purified rabbit mammary gland preparations in addition to smaller molecular weight forms (unpublished observations). Whether this larger form corresponds to a precursor that is processed or partial degradation of the PRL receptor occurring during membrane preparation and incubation with the labeled hormone remains to be clarified.

As a general concept, cytoplasmic domains of membrane receptors are responsible for the transduction of hormonal messages inside the target cells. For PRL, the present data may indicate that the two types of receptors differing in their cytoplasmic domain may trigger different biological actions of PRL. This may explain in part the origin of the diversity of biological effects of PRL (1). The first type of receptor identified in rat liver possesses a particularly short cytoplasmic domain (57 amino acids), which is reminiscent of the structural arrangement of transferrin (23) and low density lipoprotein (24) receptors that act primarily as transporters

for transferrin and cholesterol, respectively. In fact PRL has been detected in numerous biological fluids (milk, cerebral spinal fluid, amniotic fluid, and semen) suggesting that receptors localized in an epithelial barrier may be involved in the transport of the hormone from blood to biological fluids. The second type of PRL receptor that possesses a longer cytoplasmic domain has been identified in the present study from the mammary gland, an organ where PRL is primarily involved in the control of gene expression. This extended cytoplasmic domain shows several regions of high similarity with GH receptor but does not show similarity with any other hormone receptor for which the mechanism of transduction has been established. No consensus sequence for a tyrosine kinase domain nor any potential phosphorylation site was found. Interestingly, the identical location of several highly conserved sequences between PRL and GH receptors in the cytoplasmic domains suggests their potential involvement in signal transduction of both hormones and may be of potential interest in elucidating their mechanism of action. The availability of PRL receptors and GH receptor cDNAs should provide tools to develop functional cellular systems to facilitate a better understanding of the mechanism of action of PRL and GH and may clarify whether the structural features common to both receptors are of functional significance.

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