Ligand-Induced Receptor Dimerization May Be Critical for Signal Transduction by Choriogonadotropin

Navreena Grewal, Sushma Nagpal, Gayatri B. Chavali, Subeer S. Majumdar, Rahul Pal, and Dinakar M. Salunke
National Institute of Immunology, New Delhi 110 067, India

ABSTRACT A mechanism of signal transduction by human choriogonadotropin (hCG) has been proposed. Competitive inhibition of the binding of hCG to its receptor by the serine protease inhibitors led to the identification of local structural homology of an extracellular region of the receptor with the reactive site loop of chymotrypsin inhibitor. Synthetic peptides from the extracellular domain of luteinizing hormone-choriogonadotropin (LH/CG) receptor, rationally designed on the basis of this homology, were found to affect hormone-receptor binding and bioactivity. A receptor peptide incorporating one complete structural unit of the leucine-rich repeats motif of the extracellular domain of the receptor significantly increased hCG-receptor binding in a dose-dependent manner. However, the testosterone production in a Leydig cell bioassay was inhibited in the presence of this peptide. The agonistic effect on the hCG-receptor binding and the antagonistic effect on the testosterone production of a receptor peptide suggests the possibility of more than one quasi-equivalent receptor-binding site on the hormone. Hormone-induced receptor oligomerization may therefore be involved in the mechanism of signal transduction by hCG.

INTRODUCTION

The recognition and binding of ligands such as hormones and growth factors to specific cell surface receptors are the prerequisites for their functions. Interaction of luteinizing hormone (LH) and choriogonadotropin (CG) with the LH/CG receptor on gonadal target cells is the first step in the cascade of events associated with their endocrine actions. This interaction triggers a signal, mediated by membrane-associated intracellular G-proteins (Gilman, 1984), leading to the increase in adenylyl cyclase activity and followed by the corresponding increase in cAMP concentration. The increase in cAMP levels eventually results in the synthesis and secretion of steroid hormones (Dufau et al., 1980). A large number of other ligands like neurotransmitters and neuromodulators, in addition to gonadotropins, bring about changes in the cellular metabolism by interaction with receptors that are coupled to intracellular effector enzymes via G-proteins. In recent years, significant understanding of the means by which ligands trigger the cellular responses has been achieved at the structural level (Young, 1992; Sprang and Bazan, 1993).

The crystallographic structure of hCG has been determined at high resolution by two independent groups (Lapthorn et al., 1994; Wu et al., 1994). The crystal structure of placental ribonuclease inhibitor, a member of the leucine-rich repeats family of proteins to which LH/CG receptor belongs, has also been determined (Kobe and Deisenhofer, 1993). Subsequently, a knowledge-based model of the homologous domain of the gonadotropin receptors (Kajava et al., 1995) has been proposed. These high-resolution structural analyses have triggered interest in exploring the mechanism by which the information is transmitted into the cell upon binding of hCG to the membrane-associated LH/CG receptor. Several independent models for hCG-receptor recognition have been proposed (Moyle et al., 1995; Jiang et al., 1995; Grewal et al., 1994). However, these models do not exhibit any mutual agreement. We have experimentally analyzed the hCG-receptor binding by using designed synthetic peptides from the extracellular domain of the LH/CG receptor. These studies shed light on the possible mechanism of signal transduction by hCG.

EXPERIMENTAL PROCEDURES

Materials

All of the chemicals, including the Fmoc amino acid derivatives, were obtained from Nova Biochem (Laufelfingen, Switzerland). The solvents/reagents for cleavage were procured either from Sigma Chemical Co. (St. Louis, MO) or from Fluka Chemie AG (Buchs, Switzerland). The high-performance liquid chromatography-grade acetonitrile and other solvents for purification were obtained from SD Fine Chemical (Bombay, India). hCG with a biological activity of 11,370 IU/mg was procured from IBSA (Lugano, Switzerland). ^{125}I and 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-Gen) was purchased from Amersham International (Buckinghamshire, England). Aprotinin, Tris(hydroxymethyl) aminomethane (Tris), sodium azide, and bovine serum albumin (BSA) were procured from Sigma Chemical Co. or Boehringer Mannheim (Mannheim, Germany). The reagents for buffer preparations were procured from E. Merck (Bombay, India).

Peptide synthesis

The peptide synthesis was carried out by solid-phase methodology, applying the standard Fmoc chemistry by LKB Biolynx 4175 semiautomatic synthesizer on Novasyn KA resin. The peptides were cleaved from the resin, and the crude peptides were then purified by reverse-phase high-

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Address reprint requests to Dr. Dinakar M. Salunke, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110 067, India. Tel.: 91-11-6189622, ext. 386; Fax: 91-11-6162125; E-mail: dinakar@nii.ernet.in.

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performance liquid chromatography (Waters Prep LC3000 System) on a preparative Waters Deltapak C18–100 Å column, using a TFA/acetonitrile solvent system. The purity was confirmed by N-terminal sequencing on a Applied Biosystems protein sequencer (model 470A).

Radio-receptor assay

The peptides were evaluated for their effect on the binding of radiolabeled human choriogonadotropin ([^{125}I]hCG) with particulate Leydig cell LH/CG receptor by radio-receptor assay. The hCG was labeled with [^{125}I] by the Iodo-Gen method (Fraker and Speck, 1978). Testes of adult Wistar rats were used as the source of plasma membranes, prepared by the method of Dighe and Moudgal (1983). The receptor-enriched membrane fractions were incubated for 2 h at 37°C with varying dilutions of either the peptides or the aprotinin in the presence of [^{125}I]hCG (100,000 cpm). The radioactivity in the pelleted complexes of the hormone-receptor was counted in a LKB 1260 MultiGamma II Gamma counter. The maximum binding (100%) corresponds to the binding of [^{125}I]hCG to the receptors in the absence of cold hCG or any other ligand.

Leydig cell bioassay

The dose-dependent effect of the 38-mer peptide on the hCG activity was estimated using mouse Leydig cell preparation in vitro (Van Damme et al., 1974). Leydig cells produce testosterone upon stimulation with LH or hCG. In brief, semipurified preparations of mouse Leydig cells were stimulated with various doses of hCG in the range of 3.9–1000 pg/ml. The concentration corresponding to the midportion of the standard curve (125 pg/ml) was used for stimulation of testosterone production by Leydig cells in the presence of the peptide. For the assay, varying concentrations of the 38-mer peptide were added in duplicate to assess its effect on testosterone stimulation by a constant amount of hCG (125 pg/ml). Tubes containing cells and hCG alone served as the 100% stimulation in the absence of the peptide. The testosterone produced was assayed by radioimmunoassay with a kit provided by World Health Organization. Bioneutralizing capacity of the peptide was estimated by its inhibition of testosterone production.

Computer modeling

The ALIGN program (available with a NBRF sequence data package on μVAXII) was used for sequence comparisons (Dayhoff et al., 1983). The BIOSYM molecular modeling software INSIGHTII (Biosym Technologies) was used on a INDIGO$_2$ workstation (Silicon Graphics) for all aspects of model building, analysis, and display of structural data. Knowledge-based modeling of the receptor was carried out with the HOMOLOGY module based on the TSH receptor coordinates (Kajava et al., 1995). The model was refined in an AMBER force field (Weiner et al., 1986) using energy minimization. A distance-dependent dielectric constant of 4 was used, and no cross-term energies were included. The DOCKING module in BIOSYM was used to build the model of the hCG-receptor complex.

RESULTS AND DISCUSSION

Structural relationship between LH/CG receptor and serine protease inhibitors

The proteinaceous inhibitors of serine proteases inhibit the binding of gonadotropins to their receptors. Iodinated hCG has been shown to be displaced in a dose-dependent manner from the plasma membrane preparations of rat ovaries by aprotinin (Wilks and Hui, 1987). Aprotinin is a single polypeptide chain of 58 amino acids with inhibitory specificities for serine proteases like trypsin and chymotrypsin. The dose-dependent inhibition of radiolabeled FSH binding to the soluble receptor as well as receptor-enriched membrane fractions by aprotinin has also been shown (Grasso and Reichert, 1989). The serine protease inhibitors were earlier reported to block hormonal activation of adenylate cyclase (Richert and Ryan, 1977) in a variety of tissues. The antagonistic effect of the proteinaceous inhibitors of serine proteases on hCG-receptor binding could have structural implications. Therefore, radioreceptor assays were carried out in the presence of varying concentrations of aprotinin, and it was confirmed that aprotinin competitively inhibits hCG-receptor binding in the millimolar concentration range (Fig. 1). The curve shows binding of [^{125}I]hCG in the presence of various concentrations of aprotinin. The bound [^{125}I]hCG was ~62% with respect to the control at the aprotinin concentration of 0.1 mM. When the concentration was increased to 1.5 mM, the hormone binding decreased to ~18%. For comparison, the displacement of [^{125}I]hCG by cold hCG, which takes place in the micromolar concentration range, is also shown.

The antagonistic effect of a serine protease inhibitor in hCG-receptor binding could imply that the inhibitor binds to the receptor-binding site of the hormone or to the hormone-binding site of the receptor, thereby causing steric inhibition. Because aprotinin interferes primarily in the recognitive process, it may mimic the functional determinants of either the hormone or the receptor. It has been suggested that the chymotrypsin active site has similarity with the

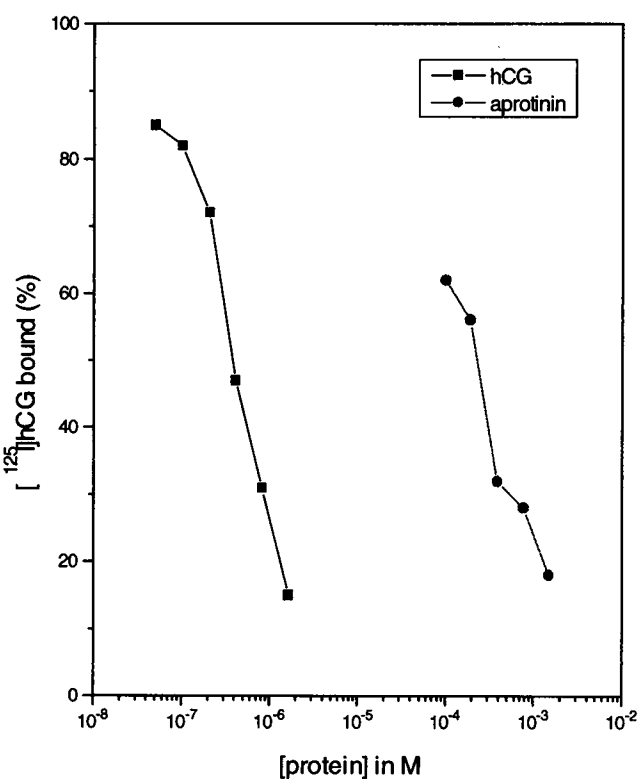

FIGURE 1 Competitive inhibition of [^{125}I]hCG binding to LH/CG receptor-enriched rat luteal membranes by increasing concentrations of cold hCG and aprotinin.

functional determinant of hCG (Willey and Leidenberger, 1989). Therefore it was expected that the reactive site of inhibitors of chymotrypsin may have analogy with the hormone-binding site of the LH/CG receptor. This receptor, like other G-protein-coupled receptors, is organized into distinct structural domains for hormone binding and effector functions. The hormone binds primarily in the extracellular domain of the receptor (Xie et al., 1990; Moyle et al., 1991). The reactive site of serine protease inhibitors is made up of an exposed loop of a characteristic conformation (Laskowski and Kato, 1980). The crystal structures of a large number of different proteinaceous inhibitors of serine proteases have been deposited in the Brookhaven Protein Data Bank (Bernstein et al., 1977). Although the inhibitors are unrelated in terms of the sequence and the overall structures, the reactive sites show significant structural similarity, which correlates with the overlapping specificity of these inhibitors.

The sequence alignment between the LH/CG receptors from various species and the inhibitors of serine proteases led to the identification of two stretches from the receptor sequence (Grewal et al., 1994), one long and one short, with one continuous stretch of chymotrypsin inhibitor-2 (CI-2). The porcine receptor sequences from 137 to 167 and from 99 to 104 match well with the CI-2 sequence from 46 to 83. The corresponding regions of the receptors from the rest of the species also show significant homology, as the receptors themselves are highly homologous. The alignment of LH/CG receptor sequences from the four species with the reactive site stretches of CI-2 and the closely related eglin C is shown in Fig. 2 *A*. There are ~50% chemically similar residues between CI-2 and the receptor stretches.

The model of the leucine-rich region of the extracellular domain of the LH/CG receptor (Fig. 2 *B*) was based on the leucine-rich repeats pattern-matching with placental ribonuclease inhibitor crystal structure (Kobe and Deisenhofer, 1993), and was obtained (see Experimental Procedure) by transcribing the homologous thyrotropin receptor model (Kajava et al., 1995) to the LH/CG receptor sequence. The comparison of the backbone conformation (McPhalen and James, 1987) of the reactive site loop of CI-2 (Fig. 2 *C*) and the corresponding segment of the LH/CG receptor model shows remarkable correspondence. Although the overall structures of these two independent proteins are very different, there is obvious local similarity between the two proteins in the homologous sequence segments (Fig. 2 *A*). Thus the reactive site loop of the serine protease inhibitors may actually be a fortuitous mimic of a small portion of the LH/CG receptor.

Designed peptides affect receptor binding and testosterone production by hCG

The similarity of the reactive site loop of CI-2 to a receptor segment implies that the synthetic peptides from this region of the receptor may also show antagonistic interference in hCG-receptor binding. A total of four peptides were designed. The relative positions of these peptides with respect to the receptor sequence are shown in Fig. 3 *A*. The longest peptide (38-mer) consists of two independent receptor stretches, Leu^{137}–Val^{167} and Asn^{99}–Lys^{104}, which are joined via an alanine residue to maintain spatial juxtaposition. The other peptides were 25 (25-mer), 24 (24-mer), and 16 (16-mer) residues long. The 25-mer (Pro^{143}–Val^{167}) includes residues corresponding to the core loop of the inhibitor. The 24-mer (Ile^{140}–Gly^{163}) and 16-mer (Lys^{158}–Val^{167} and Asn^{99}–Leu^{103}) predominantly correspond to the N-terminal and the C-terminal parts of the 38-mer peptide. In addition, an entirely unrelated 33-residue peptide of a length comparable to that of the receptor peptides was also used as a negative control (Fig. 3 *B*).

The effects of these peptides on the binding of hCG to its receptor were studied by radio-receptor assay (Fig. 3 *C*). The 38-mer showed a significant dose-dependent increase in the binding of radiolabeled hCG to the receptor, reaching 150% at 1 mM concentration. The 25-mer that structurally corresponds to the core loop also exhibited enhanced [^{125}I]hCG binding to the receptor, although this was slightly lower, reaching 126% at this concentration. However, the peptides covering the N-terminal (24-mer) and the C-terminal (16-mer) regions of the 38-mer showed no effect on hCG-receptor binding. No change in binding was observed with the nonspecific peptides as well. The structural relationship with the serine protease inhibitor was used as the receptor peptides were designed. Therefore, as in the case of aprotinin, a decrease in the hCG-receptor binding was expected in the presence of these peptides. Yet the observed dose-dependent increase still suggests a role for this receptor loop in hormone-receptor recognition.

The 38-mer receptor peptide, however, does not exhibit any dose-dependent enhancement of testosterone production in the Leydig cell bioassay. On the contrary, an inverse effect was observed. The assay was performed in the presence of different peptide concentrations. The hCG concentration used in this assay corresponds to the midpoint of the dose-response curve of hCG-induced testosterone production by mouse Leydig cells (Fig. 4 *A*). In hCG-stimulated Leydig cells, increasing concentration of 38-mer peptide resulted in a proportional inhibition of testosterone production (Fig. 4 *B*). The testosterone production remained negligible up to the peptide concentration of 0.5 mM. However, with a further increase in the concentration of the peptide there was a dose-dependent inhibition of testosterone production leading to more than 80% inhibition at a peptide concentration of 1.5 mM. The 24-mer and the nonspecific peptide do not show any dose-dependent effect on the testosterone production.

Thus the Leydig cell bioassay and the radio-receptor assay, involving a synthetic peptide designed on the basis of structural analogy with the proteinaceous serine protease inhibitors, suggest that the receptor segments corresponding to this peptide have a role in hCG recognition. The receptor peptide (38-mer) showed dose-dependent inhibition of hCG

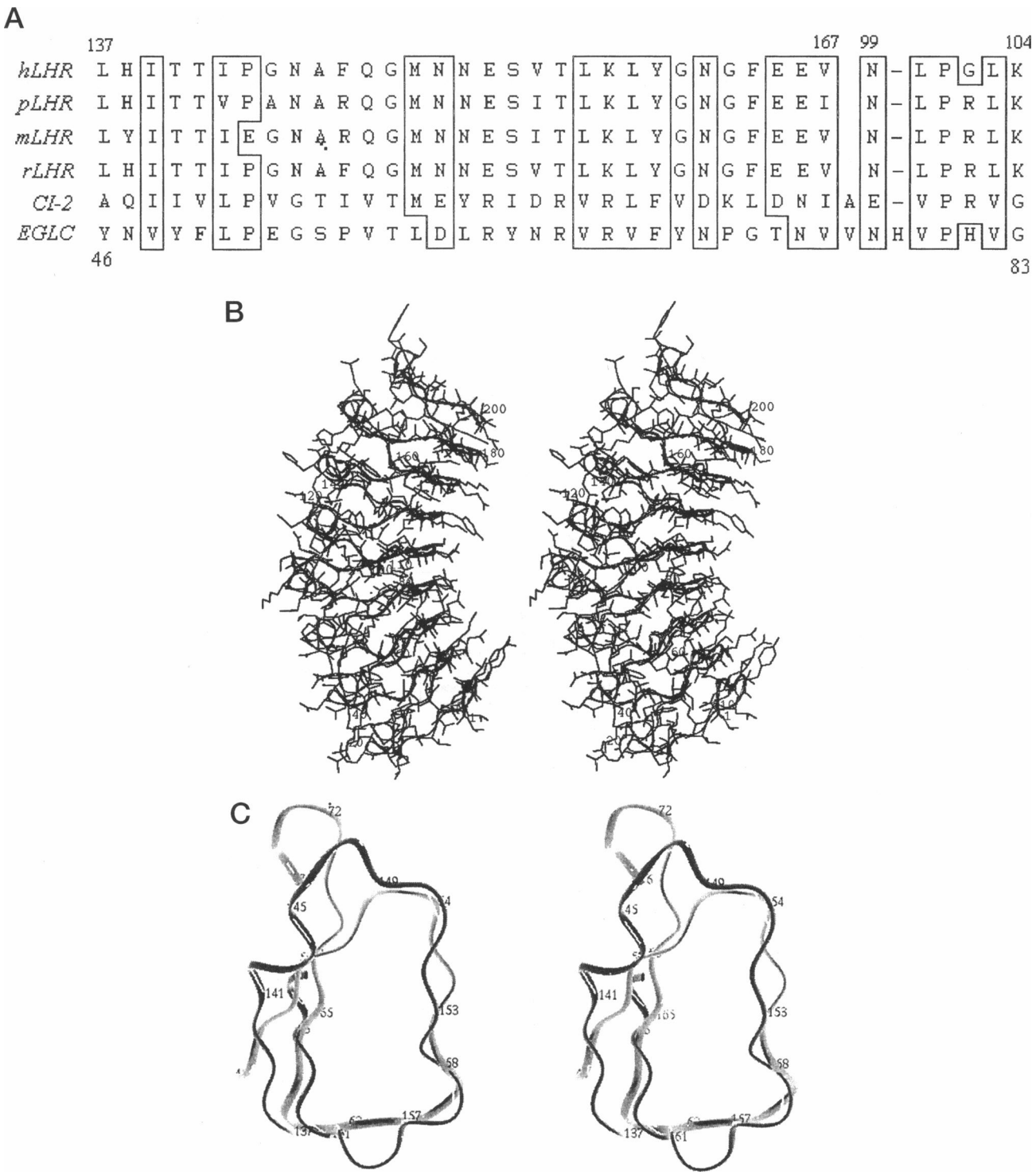

FIGURE 2 (*A*) Sequence alignment of the reactive site segments of the serine protease inhibitors with the extracellular domains of the human (hLHR), porcine (pLHR), mouse (mLHR), and rat (rLHR) LH/CG receptors. The serine protease inhibitors are chymotrypsin inhibitor (CI-2) and eglin C (EGLC). The alignment of the equivalent residues is highlighted. The numbering corresponds to porcine receptor (*top*) and CI-2 (*bottom*) sequences. (*B*) A stereo diagram showing the model of the leucine-rich domain of the LH/CG receptor. (*C*) A stereo diagram showing the superimposition of the backbone conformations of the reactive site loop of CI-2 and the LH/CG receptor segment that showed sequence similarity with CI-2.

bioactivity. However, the radio-receptor assay in the presence of this peptide showed enhanced binding of hCG to the particulate LH/CG receptor in a dose-dependent manner. On the other hand, aprotinin neutralizes the bioactivity of hCG as well as competitively inhibiting hCG-receptor binding (Wilks and Hui, 1987; see also Fig. 1 *B*).

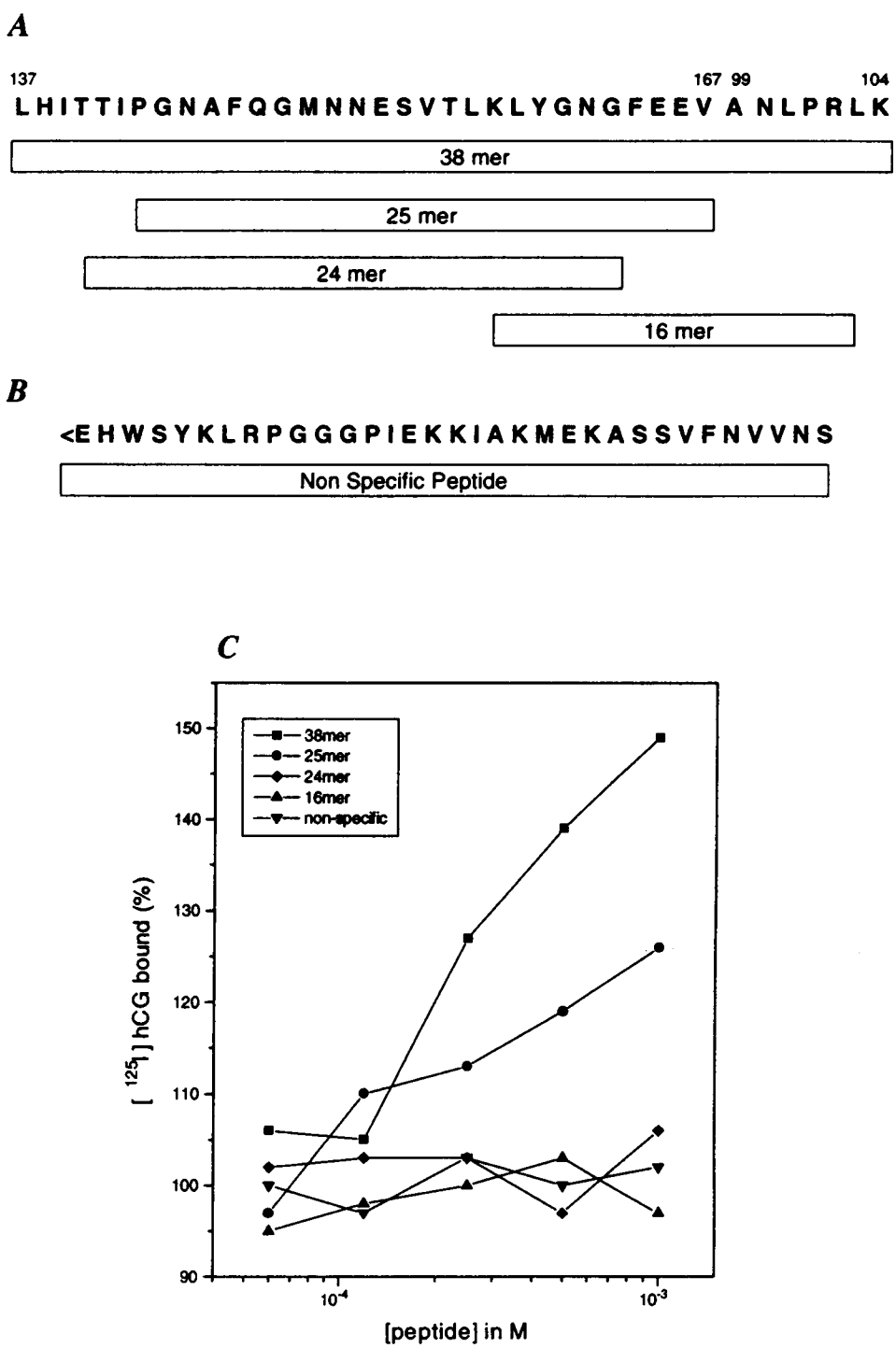

FIGURE 3 (*A*) The sequences and the relative positions of the four peptides corresponding to the porcine LH/CG receptor designed for the receptor binding and bioneutralizing activity assays. (*B*) The sequence of a 33-residue nonspecific peptide used as a negative control for receptor binding and bioneutralizing activity assays. <E represents pyroglutamic acid. (*C*) Percentage binding of [^{125}I]hCG to the particulate rat Leydig cell membrane preparations in the presence of varying concentrations of the four receptor peptides and the nonspecific peptides.

Ligand-induced dimerization

The unexpected behavior of the LH/CG receptor peptides in terms of the receptor binding and testosterone production by hCG can be interpreted if the signaling mechanism of hCG involves ligand-induced receptor oligomerization. The ligand-induced receptor oligomerization has been recognized to be a key event in signal transduction by many membrane-bound receptors (Heldin, 1995; Yarden and Schlessinger, 1987; Elberg et al., 1990; Yarden and Ullrich, 1988). Depending on the receptor and the subunit nature of the ligand, homomeric as well as heteromeric association-forming oligomers of different stoichiometric compositions have been observed (Young, 1992). The first structural evidence supporting this phenomenon was provided by the crystal structure of growth hormone complexed with its receptor (de Vos et al., 1992). The structure reveals that one molecule of the growth hormone binds to two identical molecules of the receptor, each recognizing a different portion of the hormone. The nonsymmetrical binding of the hormone to the

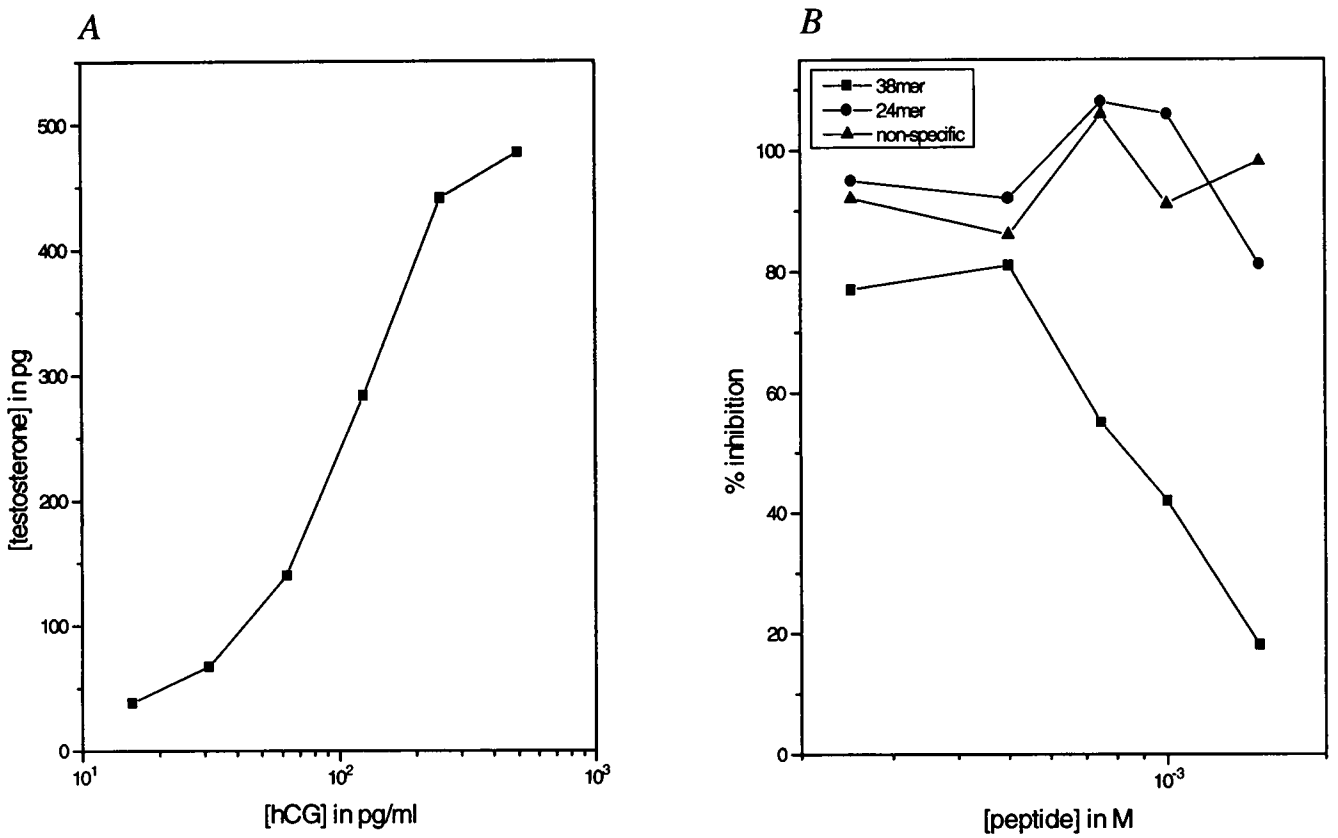

FIGURE 4 (*A*) Dose-response curve of hCG-stimulated testosterone production. The actual amounts of the testosterone produced by Leydig cells upon stimulation with various doses of hCG are plotted. (*B*) Testosterone production by mouse Leydig cells in the presence of hCG (200 μl of 125 pg/ml) and increasing concentration of the three peptides, namely, 38-mer, 24-mer, and the nonspecific peptide. One hundred microliters of various peptide concentrations were added to each tube containing Leydig cells and hCG. The incubation of this mixture was carried out at 37°C for 3 h. One hundred percent on the *y* axis represents testosterone produced by Leydig cells in the absence of any peptide.

receptor is sequential, with the two sites having different binding affinities. The crystal structure of tumor necrosis factor-soluble receptor complex (Banner et al., 1993) depicts ligand-dependent aggregation induced by a trimeric ligand. In a symmetrically organized structure of the complex, each of the three bound receptor molecules binds at the site created by the interface of two subunits. The enhanced binding but depleted receptor activation by hCG in the presence of the designed peptide can be explained, if such a mechanism of receptor activation by hCG is hypothesized. In this case, the quasiequivalent multiple binding sites on the ligand may result in receptor association bringing neighboring receptor molecules in the membrane bilayer into close proximity and the generation of new intracellular sites for effector functions.

The receptor peptides, which enhance binding of hCG to its receptor at millimolar concentrations, could bind to the hormone while simultaneously allowing the binding of a receptor molecule on an independent site. In a radio-receptor assay in which a [^{125}I]hCG is pelleted out with the Leydig cell membrane preparations containing LH/CG receptors, reduction in the stoichiometry of the ligand-receptor binding would lead to an increase in the radiolabeled hormone available for binding to the receptor on the membrane. In these circumstances, an increase in the bound [^{125}I]hCG with increasing concentrations of the peptide can be anticipated. However, not completing the stoichiometry of the ligand-receptor complex would imply that the cytosolic sites for effector functions may not be generated, leading to the abortion of the signal transduction cascade. Thus enhancement in [^{125}I]hCG binding to the receptor should not be accompanied by testosterone production, which should actually decrease as a function of the increasing concentration of the peptide, if the mechanism of signal transduction by hCG is achieved by ligand-induced oligomerization. The observed reduction in the testosterone production (Fig. 4 *B*) with respect to the increase in the concentration of 38-mer precisely follows this logic.

The hCG molecule is a heterodimer made up of two nonidentical polypeptide chains. However, the two subunits are topologically equivalent and are related by a pseudo-twofold symmetry (Lapthorn et al., 1994; Wu et al., 1994). In addition, both of the subunits of the hormone adopt a cystine-knot topology (McDonald and Hendrickson, 1993) common to the growth factors like nerve growth factor (NGF) (McDonald et al., 1991)), tissue growth factor (TGF)

β (Daopin et al., 1992), and platelet-derived growth factor (PDGF) B (Oefner et al., 1992). These growth factors are known to be homodimers and have been shown to induce receptor dimerization upon binding. Besides the topological similarities to the growth factors, hCG has also been suggested to have growth-factor-like functional characteristics (Lazar et al., 1995; Yoshimura et al., 1994). It is therefore possible to conceive of two quasiequivalent binding sites on the hormone, considering the substantially asymmetrical nature of the hCG dimer, as has been observed in the case of growth hormone (de Vos et al., 1992). Consequently, the effective number of available membrane-associated receptor molecules for the radiolabeled ligand increases. The binding of peptides to the radiolabeled hCG facilitates subsequent binding of the hormone to the receptor, but prevents oligomerization, henceforth resulting in an enhanced radioactivity in the assay. On the other hand, a comparatively large and substantially different molecule like aprotinin, even while partially sharing topology with the receptor peptide, could sterically prevent the hormone-induced receptor association. In the absence of any other ligand, the receptor alone will saturate the binding sites of the hormone.

A model of the complex of LH/CG receptor and hCG, consistent with the hCG-induced receptor dimerization, was constructed in light of the above data and the knowledge of the three-dimensional structures of hCG (Lapthorn et al., 1994; Wu et al., 1994) and the gonadotropin receptors (Kajava et al., 1995). The approximate dimer symmetry and the experimental knowledge of the segments of hCG (Ryan et al., 1987; Charlesworth et al., 1987; Reed et al., 1991; Merz and Dorner, 1985) and the receptor (Roche et al., 1992; present study) involved in the binding were utilized in this model building. A receptor molecule was first docked with the hormone by using the binding site segments as guide, and the docking was optimized by monitoring the energy of interaction. The approximate twofold symmetry of hCG was used for generating the second receptor molecule from the first, which was then independently optimized again by evaluating the interaction energy. The final model of the hormone receptor complex is shown in Fig. 5. In this model, two binding sites of hCG are occupied by two independent receptor molecules. The region of the receptor modeled here corresponds only to the leucine-rich repeat region of the extracellular domain. The unmodeled regions of the receptor are schematically shown as blobs. Fig. 5 also indicates the juxtaposition of the hCG-receptor complex with respect to the membrane. As shown in the model, the two receptor molecules may interact with themselves through the transmembrane domains and the adjacent, small, non-leucine-rich regions of the extracellular domains. This would facilitate bringing together the cytosolic domains and the creation of an intracellular site for effector functions. Almost all of the repeats in the receptor model are involved in hCG binding (Fig. 5), and the segment corresponding to 38-mer covers only one of them. Thus the peptide (which is indicated in the figure by *grey ribbon*) incorporates only a fraction of the hormone-binding site. This explains the comparatively low affinity of the peptide.

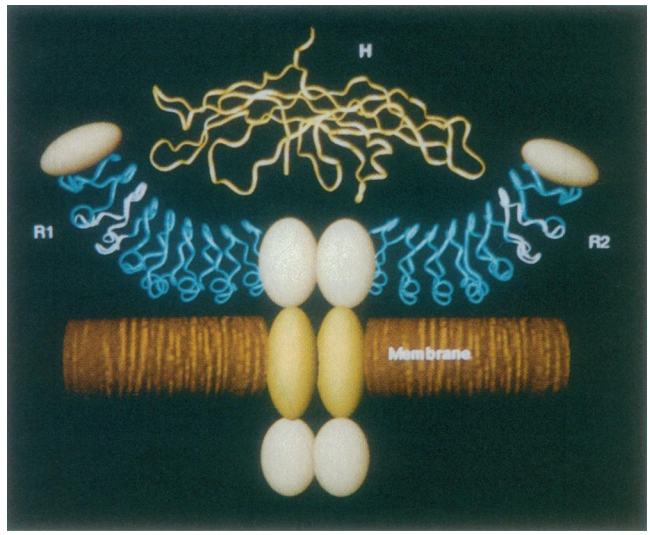

FIGURE 5 A model illustrating dimerization of the LH/CG receptor by a single hCG molecule. H, R1, and R2 correspond to the hormone and the two receptor molecules, respectively. The hormone (*orange*) and the leucine-rich region of the LH/CG receptor (*blue*) are shown as ribbons. The N- and C-terminal regions of the extracellular (*light yellow*), transmembrane (*yellow*), and cytosolic (*light yellow*) domains that do not have a leucine-rich motif are shown schematically as blobs. The 38-mer peptide is highlighted by grey ribbon in both of the receptor molecules.

Certain ligand-dependent observations have also been made regarding the mechanism of signal transduction involving LH/CG and other similar receptors that lend indirect support to the receptor dimerization model. The hCG-induced aggregation of receptor molecules on the cell surface was reported several years ago (Podesta et al., 1983, 1986). Recent studies (Maggio et al., 1993) have shown that functional intermolecular interactions also occur with the muscarinic and the adrenergic receptors. These receptors, like the LH/CG receptor, belong to the G-protein-coupled receptor family. These otherwise independent observations appear to be consistent with the results described above. It can therefore be assumed that signal transduction by hCG takes place by sequential binding of the receptors to two quasiequivalent sites, leading to receptor dimerization, as in the case of growth factors.

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