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High-Level Bacterial Expression of a Natively Folded, Soluble Extracellular Domain Fusion Protein of the Human Luteinizing Hormone/Chorionic Gonadotropin Receptor in the Cytoplasm of *Escherichia coli*

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We have expressed the extracellular domain of the human luteinizing hormone/chorionic gonadotropin (hLH/CG) receptor as a fusion protein with thioredoxin in the cytoplasm of an *Escherichia coli* strain that contains mutations in both the thioredoxin reductase and glutathione reductase genes. The chimeric protein isolated following induction of expression is purified in a soluble form and binds hCG with an affinity approximating that of native receptor. This truncated form of the receptor displays the same specificity as intact hLH/CG receptor and does not bind human follicle stimulating hormone. This cytoplasmically produced protein is expressed at levels that exceed 10 mg/L. Expression of properly folded extracellular domain of the hLH/CG receptor in the cytoplasm of *E. coli* allows the facile and economic purification of large quantities of material. This will facilitate the determination of the structure of the hormone-binding domain of the glycoprotein receptor as well as the production of epitope-specific antibodies.

Key Words: Human luteinizing hormone/chorionic gonadotropin receptor; chorionic gonadotropin; bacterial expression; fusion protein.

Introduction

The expression of mammalian proteins in *Escherichia coli* has provided an economic and facile method for producing large quantities of recombinant proteins *(1,2)*. Proteins produced in this way can be useful for biochemical studies such as structural determination as well as for assay development or the identification of pharmacologically useful compounds with high-throughput screening techniques *(3,4)*. In addition, recombinant proteins produced in non-mammalian systems can potentially be useful as therapeutic agents *(2)*.

We have been studying structural and functional aspects of the human luteinizing hormone chorionic gonadotropin (hLH/CG) receptor. We previously expressed the extracellular domain of the receptor as a fusion with the capsid protein III (*cpIII*) of filamentous phage *(5)*. The fusion phage bound hCG specifically and with comparable affinity to that of the native receptor *(5)*. The orientation of binding was also consistent with that of the native receptor *(5)*. Although the cpIII phage display system has proven to be useful in high-throughput screening protocols for antagonists of the hLH/CG receptor and other proteins (unpublished data), it is not a useful expression technique for production of large quantities of a protein. The incorporation of a single copy of the recombinant protein on the surface of the phage, on average, and limitations on the titer of phage particles greatly reduces the utility of this method as an expression technique. Alternative phage display systems such as the *cpVIII* gene of filamentous phage offers increased expression of the desired fusion protein because there are approx 2700 copies of the gene product on the surface of each filamentous phage *(6,7)*. Nonetheless, as the size of the fusion protein increases, incorporation in the capsid of the fusion product decreases. Although the extracellular domain of the hLH/CG receptor can be fused to the cpVIII gene and folded properly on the surface of the phage, <50 copies, on average, are incorporated in the capsid of a single phage (unpublished observations). These fusion phage are therefore incapable of expressing the receptor-binding domain at a level that would be feasible for producing large quantities of this protein.

Earlier attempts at expressing the extracellular domain of the hLH/CG receptor employed eukaryotic, prokaryotic, and insect cell vectors. Although baculovirus-based systems can express sufficient quantities of material, our previous attempts at expression in this system failed to produce significant quantities of material that could bind hCG with a

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high affinity. Whereas Chen and Bahl *(8)* have previously reported the production of the hormone-binding domain in *E. coli*, this material was not folded correctly and had to be refolded in vitro. This procedure is laborious and generally not useful for the isolation of large quantities of homogenous proteins. Successful isolation of the extracellular domain of the hLH/CG receptor from a chimerically expressed eukaryotic protein has been previously reported *(9)*. Nonetheless, this method of expression is relatively labor-intensive and does not provide sufficient material for detailed structural studies of the binding domain of the receptor.

To obviate the problems and limitations previously encountered with other expression techniques, we reexamined the expression of the extracellular domain of the receptor in an *E. coli* system. Studies of protein expression in *E. coli* have demonstrated that the environment in bacterial cytoplasm does not favor disulfide bond formation in cytoplasmically expressed recombinant proteins. However, the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes play a major role in the thiol-disulfide balance in the *E. coli* cytoplasm *(10,11)*. Mutants of *E. coli* in either or both of these genes tend to favor disulfide bond formation in cytoplasmically expressed recombinant proteins *(10)*. Therefore, we focused on the expression of the extracellular domain of the hLH/CG receptor in an *E. coli* strain containing mutations in these reductase pathways.

Our studies demonstrate the successful expression of the hLH/CG extracellular domain in the cytoplasm of *E. coli* as an inducible fusion protein with thioredoxin in both a *trxB* host and a *trxB/gor* host strain that is mutant in both reductases. The recombinant soluble protein is folded properly and can bind hCG specifically, with an affinity close to the native intact receptor. Furthermore, following a 3-h induction, 10–30 mg/L of fusion protein can be obtained in this expression system. The production of large quantities of the extracellular domain of the hLH/CG receptor that is folded properly will be useful for the generation of a panel of epitope-specific antireceptor monoclonal antibodies (MAbs) and structural analysis of the hLH/CG receptor by X-ray crystallography. In addition, large quantities of a soluble form of the hLH/CG receptor might be useful clinically to regulate the circulating levels of hCG for contraception or treatment of a variety of neoplasias that appear to respond to hCG in either an autocrine or a paracrine mode *(12)*.

Results

Construction of an Expression Clone Containing the Extracellular Domain of hLH/CG Receptor

To express the extracellular domain of the hLH/CG receptor in *E. coli*, we constructed a number of different expression clones. Few of these showed significant expression on induction. In particular, attempts to express the extracellular domain of the receptor in the cytoplasm of *E. coli* failed in several different strains tested. As a result, we concluded that those sequences did not fold properly or were perhaps unstable by themselves in the cytoplasm of *E. coli*. To address this problem, we fused cDNA encoding the extracellular domain of the hLH/CG receptor with a number of different proteins that were known to enhance stability and promote folding of the tethered sequences. One clone, designated LR4, expressed significant amounts of material on induction. It contained a fusion of the receptor sequences downstream of a thioredoxin gene in a pET32a(+) vector and is the source of all the recombinant material described in this article. Clone LR4 contains residues 1–336 from the mature receptor protein. These residues encompass nearly the entire extracellular domain of the receptor (*see* Fig. 1).

Expression and Purification of the Fusion Protein Encoded in LR4

To express the recombinant receptor-thioredoxin fusion protein, LR4 DNA was transformed into appropriate recipient expression strains. The two expression strains that we employed were AD494 *trxB* (DE3)pLysS (kan^R, Cm^R) and Origami *trxB gor* (DE3)pLysS (kan^R, tet^R, Cm^R). Both strains are mutant in the thioredoxin reductase locus with the Origami strain containing an additional mutation at the glutathione reductase locus. These mutations promote proper protein folding by facilitating disulfide bond formation in cytoplasmically expressed recombinant proteins in *E. coli (10)*. Strains that were wild type at both of these loci yielded lower levels of expression from LR4 following induction with isopropyl-thio-β-D-thiogalactoside (IPTG). In addition, much of this material could not be purified in soluble form.

We routinely monitored induction of expression on nonreducing sodium dodecyl sulfate (SDS) polyacrylamide gels (*see* Fig. 2A, lanes C and 1). Comparison of expression in strains AD494 and Origami indicated that the latter strain consistently expressed higher levels of recombinant protein at each time point following induction (data not shown). As a result, we chose the Origami strain of bacteria for all expression studies.

The induced fusion protein was initially harvested as aggregated material that was resolubilized in buffer containing the anionic detergent *N*-lauroylsarcosine at a final concentration of 0.25%. This material was >90% pure as determined by SDS polyacrylamide gel analysis (*see* Fig. 2A, lane 2). Further protein purification of the fusion protein and removal of the detergent was accomplished by Ni-NTA resin affinity chromatography (Qiagen). The eluted fusion protein electrophoresed as a single species. A sample silver-stained gel is illustrated in Fig. 2A, lane 3. Purified protein was soluble and could then be concentrated. Maintaining solubility of the protein at higher concentrations was facilitated by the addition of various nondetergent sulfobetaines.

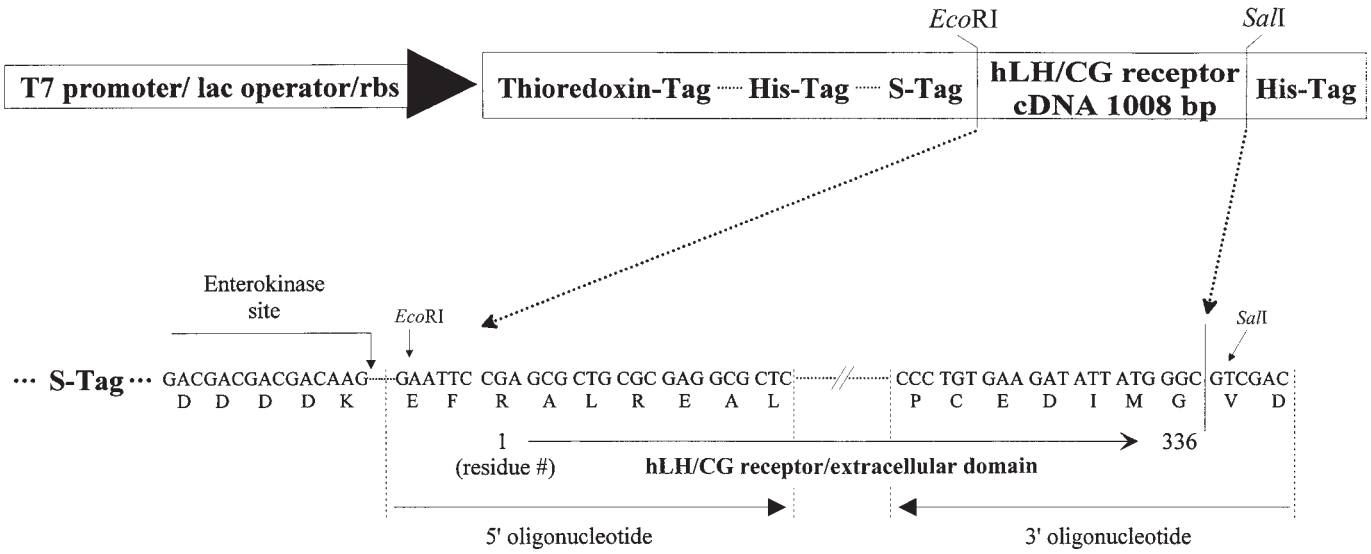

Fig. 1. Diagram of LR4 vector illustrating the construction of the LR4 vector from pET32a(+) and sequences encoding the extracellular domain of the hLH/CG receptor.

Recombinant Receptor Protein Binds Antireceptor Antibodies

To confirm that the recombinant protein expressed was the extracellular domain of the receptor, Western blot analysis was performed using polyclonal antibodies and MAbs against the extracellular domain residues of the hLH/CG receptor. The polyclonal antisera was produced in our laboratory to a denatured polypeptide containing residues from the extracellular domain of the hLH/CG receptor that had been expressed in *E. coli* and was previously described *(5)*. MAbs to the extracellular domain of the hLH/CG receptor, LHR29, were a generous gift from E. Milgrom *(13)*. The blots are displayed in Fig. 2B. They demonstrate that both polyclonal antibodies and MAbs bound a single band that migrated at the expected molecular weight; control extracts expressing the thioredoxin protein without the receptor sequences were blank (data not shown). Therefore, we can conclude that the induced fusion protein contains the extracellular domain of the hLH/CG receptor.

Affinity of Receptor-Thioredoxin Fusion Protein for hCG

To determine the affinity of the fusion protein for hCG, we developed a novel assay that utilized the His tag to capture receptor-bound hCG (*see* Materials and Methods). Briefly, purified fusion protein was incubated with ^{125}I-hCG and varying concentrations of cold hCG and receptor material were then separated from unbound labeled hCG with Ni-NTA resin. Resin-bound material was separated by centrifugation, washed, and then counted. The results of this analysis are illustrated in Fig. 3. This assay was highly reproducible and demonstrated that the affinity of the soluble receptor fusion protein for hCG was on the order of $10^{-9}M$. This is comparable to the affinity of the native receptor for hCG that was previously determined in our laboratory and by others *(14–16)*.

To study the specificity of the fusion protein for hCG, we incubated the protein with labeled hCG in varying concentrations of human follicle-stimulating hormone (hFSH). As illustrated in Fig. 3 hFSH had no effect on binding at concentrations as high as $10^{-7}M$. Therefore, we concluded that the recombinant expressed receptor domain maintains the appropriate specificity for hCG.

Receptor Fusion Protein Binds hCG in an Orientation Consistent with That of Native Hormone-Receptor Complex

To determine whether the same surface of hCG that interfaces with the native receptor also interacts with the recombinant truncated receptor, we conducted a series of experiments using epitope-specific anti-hCG MAbs that have been previously described *(17,18)*. In particular, we employed the anti-hCG MAbs B105 and B107. B105 can bind hCG while it is bound to the hLH/CG receptor and does not block binding of free hCG to receptor. Alternatively, B107 does not bind hCG when bound to receptor and blocks binding of free hCG. As a result, we tested the effect of these antibodies on binding of labeled hCG to the recombinant receptor using the same binding assay described in the preceding section. The results of this analysis are depicted in Fig. 4. They indicate that whereas B105 has little or no effect on binding of labeled hCG to the fusion protein, B107 almost completely blocks binding of the labeled hCG to the recombinant receptor. Although B105 can at times

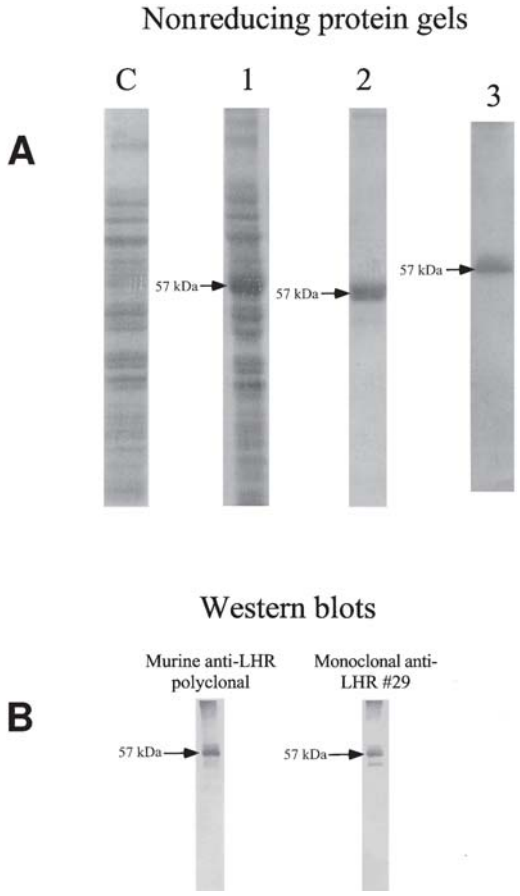

Fig. 2. Protein gels and Western blots. (**A**) Lanes from nonreducing SDS polyacrylamide gels. Lane C contains a protein extract from the host strain (Origami DE3 pLysS) induced with IPTG for 3 h. This lane serves as a control. An extract from the same strain containing the LR4 vector and induced for 1.5 h with IPTG is in lane 1 (extracts from bacteria following a 3-h induction overloads the lane of the gel). Lane 2 contains a sample of the washed and resolubilized protein aggregate. Lane 3 contains a sample of the purified protein following Ni-NTA chromatography. Lanes C, 1, and 2 are Coomassie stained whereas lane 3 is silver stained. The major protein band migrating at 57 kDa corresponds to the size of the thioredoxin-receptor fusion protein (the amino-terminal thioredoxin protein along with the S peptide and six-histidine tag is approx 18 kDa whereas the hLH receptor extracellular domain protein accounts for approx 38 kDa. All samples were boiled before loading to avoid protein aggregation in the SDS gel buffer. (**B**) A Western blot analysis of the purified fusion protein that was probed with monoclonal anti-hLH/CG receptor antibodies and also polyclonal anti-hLH/CG receptor antisera. Note that a single major species (*see* arrows) is identified in both blots. Samples for the Western blots were not boiled before loading since this reduced affinity of the antibodies to the fusion protein. As a result, there is some aggregated protein at the top of the gel. A minor band appearing on the monoclonal anti-LHR #29 Western blot is approx 51 kDa. It is much less than 1/10 *M* relative to the major 57-kDa species by densitometric analysis and is not seen on the polyclonal anti-LHR blot. This minor species is likely degraded material owing to contaminating proteases.

slightly enhance binding of hCG to the receptor, we have previously seen this phenomenon with native receptor on Chinese hamster ovary cells. These results are consistent with the hypothesis that hCG is bound in the native orientation by the receptor fusion protein.

Discussion

We have demonstrated the feasibility of expressing a properly folded extracellular binding domain of the hLH/CG receptor as a fusion protein in the cytoplasm of *E. coli*. This protein can be purified in soluble form and displays a binding affinity comparable to that of native receptor. The recombinant protein binds hCG specifically, and the binding orientation is consistent with that of the native hormone-receptor complex. Furthermore, as we have previously shown *(5)*, our results demonstrate that glycosylation of the binding domain of the receptor is not required for high-affinity interaction with hCG.

These results are the first to demonstrate that the binding domain of the hLH/CG receptor can be expressed in the cytoplasm of *E. coli* and fold properly with the appropriate disulfide bonds. In addition, we were able to achieve significant expression levels in this system. We routinely expressed 10–30 mg/L of recombinant receptor protein and were able to purify this material in soluble form. Although many recombinant proteins expressed cytoplasmically in *E. coli* are typically denatured or partially folded, we found that the reductase double mutant strain of *E. coli* that we utilized expressed our fusion protein as properly folded material.

One of the initial barriers for study of a given protein is the isolation of sufficient quantities of material. For proteins such as the glycoprotein receptors that are relatively difficult to produce and purify in large quantities, heterologous expression systems must be utilized for expression of sufficient quantities of material. Bacterial expression of eukaryotic proteins has traditionally been the most efficient and economic means for production of large quantities of a given protein. Nonetheless, this system is hampered by frequent production of insoluble and improperly folded material. Several methods for overcoming this limitation have recently been developed that include fusion to peptides that enhance solubility and folding and expression in genetically altered bacterial strains that promote proper disulfide bond formation. We have utilized these techniques to express the extracellular domain of the hLH/CG receptor in bacteria at a high level. Concurrent use of multiple methodologies as described here should be useful for the production of other proteins that have thus far been difficult to produce. To this end, we have successfully expressed the extracellular domain of the hFSH receptor in bacteria by a similar technique (unpublished data).

Our ability to express high levels of the recombinant hLH/CG receptor extracellular domain in an economical and expeditious system will enable the design of new assays to detect novel forms of hCG or hLH. Thus far, radioimmunoassays have suggested that novel glycosylated forms of hCG are associated with Down's syndrome *(19)*. This sug-

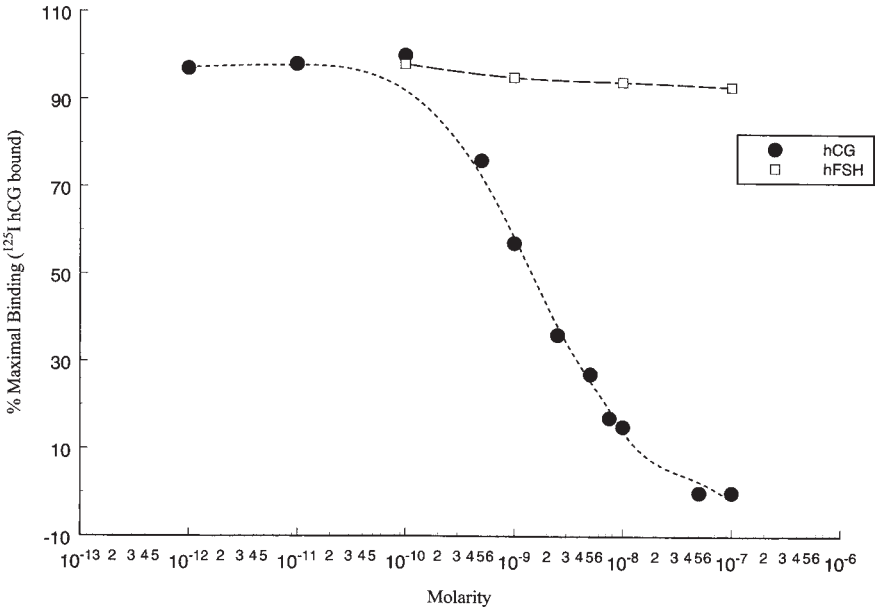

Fig. 3. Affinity of fusion protein for hCG. This affinity curve for the receptor-thioredoxin fusion protein was generated according to the techniques outlined in Materials and Methods. Fixed amounts of fusion protein and counts per minute of labeled hCG were incubated in the presence of varying concentrations of unlabeled hCG. Bound labeled hCG was isolated from the incubation mixture with Ni-NTA resin and counted. Molarity of unlabeled hCG in each incubation reaction was graphed vs the percentage of maximally bound counts as defined in Materials and Methods. hFSH was also tested in this assay to demonstrate the specificity of the fusion protein for hCG. The standard error for all points is negligible, and, therefore, error bars are omitted for clarity. Each graph point represents the mean of five binding experiments.

gestion was based on the fortuitous identification of a unique MAb that could detect these glycosylation differences. However, hCG is also secreted in a variety of other pathologic conditions, and there may be other forms that might be distinguishable on the basis of altered receptor affinities. Implementation of a simple in vitro radioreceptor assay will therefore facilitate analysis of receptor-binding affinity for hCG molecules that are secreted from a variety of malignant and benign pathologies. This could lead to a better understanding of these pathologies as well as provide new markers for diagnosis and treatment.

The availability of large quantities of a soluble form of the extracellular domain of the hLH/CG receptor will also facilitate the structural characterization of a glycoprotein hormone receptor for the first time. The material we produce will serve as a basis for crystallographic studies. In addition, large quantities of the extracellular domain of the natively folded receptor will facilitate production of a panel of epitope-specific MAbs. Thus far, most MAbs to the hLH/CG receptor were produced using receptor protein isolated from eukaryotic cells that express the receptor. The amount of material purified from these cell types is very small, and as a result, only a limited number of MAbs have been produced. Other methods for MAb production generally employ synthetic peptides. Antibodies produced using the peptide as an immunogen are not sensitive to structural nuances of the protein and, therefore, are not useful for detecting conformational changes. MAbs generated from natively folded receptor should prove useful for studying the membrane-bound structure of the receptor and examining structural variations between the unoccupied receptor and the receptor that is docked with hCG.

In summary, the ability to efficiently produce the extracellular domain of the hLH/CG receptor in a pure, soluble form will facilitate basic work on receptor structure and may have significant clinical application. Ultimately, elucidation of the quaternary structure of the ligand-binding domain of the receptor will facilitate the development of small molecule antagonists and agonists of the receptor.

Materials and Methods

Bacterial Strains

All molecular biology techniques and large-scale preparation of plasmid DNA were performed with *E. coli* strain DH5α. Expression constructs were transformed into either strain AD494 *trxB* (DE3)pLysS (kan^R, Cm^R) or Origami *trxB gor* (DE3)pLysS (kan^R, tet^R, Cm^R). Both strains were acquired from Novagen (Madison, WI) and are mutant at the thioredoxin reductase locus (*trxB*). The Origami strain is also mutant at the glutathione reductase locus and is designated *gor*. For expression of fusion constructs, the strains were freshly transformed and colonies were picked directly from the transformation plates into growth media for expression. Passing or freezing transformants leads to diminished expression from the bacterial population. Strains

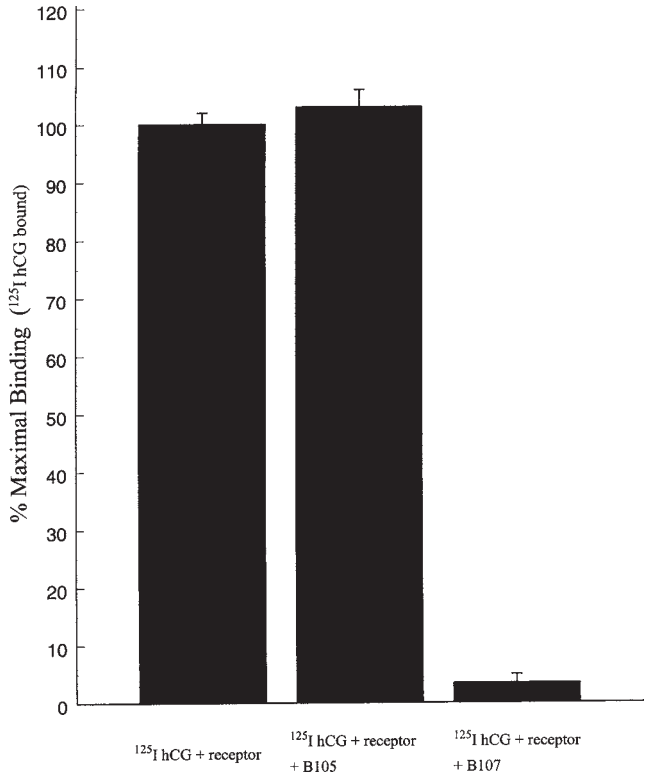

Fig. 4. Effect of anti-hCG MAbs on hCG binding to fusion protein. To determine that hCG binds the receptor-thioredoxin fusion protein in the correct orientation, we tested the effect of anti-hCG B105 and B107 on binding of labeled hCG to fusion protein. B105 can bind hCG while bound to the native receptor and does not interfere with binding of labeled hCG to fusion protein. Alternatively, B107 blocks binding of hCG to native receptor and blocks binding of labeled hCG to the fusion protein. We conclude, therefore, that these results are consistent with the hypothesis that hCG is bound by the fusion protein in an orientation similar to that of the native hormone-receptor complex.

were grown in standard Luria-Bertani (LB) medium for expression. Richer media formulations such as SOC media lead to poorer expression yields of soluble protein.

Gonadotropin Preparations

The preparations of hCG (urinary hCG CR127) used in this study have previously been described *(20,21)*. The CR127 preparation of hCG is the widely distributed reference preparation and was generously provided by Dr. Steven Birken *(21)*. hFSH was acquired from the National Pituitary Agency (National Institutes of Health).

Molecular Biology

All enzymes for recombinant DNA were purchased from New England Biolabs. DNA primers for polymerase chain reaction (PCR) were synthesized by the Columbia University Core Laboratory. The 5' primer introduced an *Eco*RI site in the same frame and adjacent to the receptor sequence whereas the 3' primer introduced an in-frame *Sal*I site (Fig. 1). PCR reactions were performed with Vent DNA polymerase (New England Biolabs), and all products of the reactions were sequenced to ensure that no mutations were introduced during the amplification process. Ligation reactions were transformed into DH5α, and DNA clones were grown in DH5α for large-scale preparation of plasmid. Transformations of DH5α and the expression strains AD494 and Origami were performed according to standard techniques with calcium chloride.

Construction of Thioredoxin Fusion Clone

The expression vector was pET32a(+) (Novagen). DNA encoding the extracellular domain of the hLH/CG receptor was isolated by PCR of a full-length clone of the hLH/CG receptor (kindly provided by Dr. Aaron Hsueh). Using primers with engineered 5' *Eco*RI and 3' *Sal*I sites, a cDNA encoding residues 1–336 of the receptor was amplified by PCR. The amplified product contained in-frame 5' *Eco*RI and 3' *Sal*I sites. This was inserted into the polylinker cloning site of the pET32a(+) vector from Novagen (Fig. 1). The resulting construct has the receptor sequences fused downstream of the thioredoxin gene. In addition, the insertion generates a frameshift such that the 3' residues at the carboxy terminus encoded in this clone are equivalent to those encoded by the pET32b(+) clone. These terminal residues in pET32b(+) comprise a second His tag. Therefore, this fusion clone encodes a second His tag at the 3' end with the other tag sandwiched between the thioredoxin gene and the receptor sequences (Fig. 1). The clone utilized for all expression studies was designated LR4.

Expression of Fusion Protein

For expression in AD494, colonies were inoculated into 10 mL of LB medium with kanamycin (50 μg/mL) and ampicillin (75 μg/mL) and grown overnight. The fresh overnight culture was diluted 1:100 into LB medium with 50 μg/mL of ampicillin and grown to an optical density of 0.5 at 600 nm. The culture was then induced with 1 m*M* IPTG for 3 h, and the bacteria were harvested by centrifugation. Induction of expression was monitored on nonreducing SDS polyacrylamide gels (Fig. 2A, lanes C and 1). Bacterial pellets were then frozen and stored at –20°C. Expression in the Origami strain was essentially the same except that the overnight culture was grown in the presence of tetracycline (25 μg/mL) in addition to ampicillin and kanamycin.

Purification of Expressed Fusion Protein

Frozen bacterial pellets were resuspended in 1 mL of Bugbuster reagent (Novagen)/50 mL of bacterial culture with the addition of 20 μg/mL of lysozyme. The suspended cells were allowed to sit on ice until lysis was complete and the suspension became viscous. The lysed cells were incubated with 25 U of Benzonase nuclease (Novagen)/mL. The mixture was incubated on ice until the viscosity disappeared. Aggregated protein was collected by further incubation of the material on ice for either 2 to 3 h or overnight at 4°C followed by centrifugation. Control pellets from an induced pET32a(+) clone in the Origami strain did not

yield any aggregated material following lysis and nuclease treatment. The protein aggregate was washed with buffer twice and collected by centrifugation. The protein pellet was resuspended in 10 m*M* $NaHPO_4$/60 m*M* NaCl (pH 8.0) at 1 mL/mL of suspended cells. *N*-Lauroylsarcosine was added at a final concentration of 0.25%, and the material was mixed well by pipetting up and down through the tip of an Eppendorf pipet to help disperse the aggregated protein. The resuspended protein was allowed to sit on ice until the solution clarified and solubilization was complete. If solubilization was not complete the solution was either incubated for 10 min at 37°C or diluted twofold and additional *N*-lauroylsarcosine was added to obtain a final concentration of 0.25%. Incomplete initial solubilization at 0.25% *N*-lauroylsarcosine generally indicated that bacterial expression exceeded 20 mg/L, and twofold dilution of the solution led to complete solubilization at 0.25% *N*-lauroylsarcosine in all cases. At this point the recombinant protein was >90% pure, as determined by SDS polyacrylamide gel analysis (Fig. 2A, lane 2).

For further protein purification and to remove the detergent, the solubilized material was further purified on an Ni-NTA resin column (Qiagen). Solubilized fusion protein in 0.25% *N*-lauroylsarcosine was diluted 10-fold in 50 m*M* $NaHPO_4$/300 m*M* NaCl (pH 8.0) for a final detergent concentration of 0.025%. This material was passed through an Ni-NTA resin column (Qiagen), and the flow-through was recirculated through the resin five times. The resin was washed with 5 vol of 50 m*M* $NaHPO_4$/300 m*M* NaCl (pH 8.0) followed by the same buffer with the addition of 10 or 20 m*M* Imidiazole (pH 8.0) for the second and third washes, respectively. The bound fusion protein was eluted with three column volumes of 50 mM $NaHPO_4$/300 m*M* NaCl/250 m*M* imidazole (pH 8.0). The eluted material was exhaustively dialyzed in 10 m*M* $NaHPO_4$ (pH 8.0).

The eluted fusion protein electrophoresed as a single species on a nonreducing SDS polyacrylamide gel. A sample silver-stained gel is illustrated in Fig. 2A, lane 3. Purified protein was soluble and could then be concentrated. Maintaining solubility of the protein at higher concentrations was facilitated by the addition of various nondetergent sulfobetaines.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the procedure described by Laemmli and Favre *(22)* and Laemmli *(23)*. The sample buffer contained 125 m*M* Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.01% bromophenol blue. Gels were 10% polyacrylamide and either Coomassie blue or silver stained according to established techniques *(24)*.

Western Blot Analysis

Following electrophoresis, the proteins in the gel were transferred to nitrocellulose paper using a variation of the methods of Towbin et al. *(25)* and Burnette *(26)*. After blocking in 5% bovine serum albumin (BSA), 0.01 *M* Tris-HCl, and 0.15 *M* NaCl (pH 7.6) (BSA-TBS), the paper was incubated overnight with antibody diluted in BSA-TBS (at concentrations of approx 3 to 4 μg of antibody/mL for MAbs and using a dilution of 1:500 of anti-hLH/CG receptor antiserum for polyclonal antibodies). The binding of the primary antibodies was visualized as previously described *(17)*.

MAbs and Polyclonal Antibodies

All MAbs used in these studies have been previously described *(13,17,18)*. B105 binds both intact hCG and free β-subunit, but it does not block hCG from binding to the receptor and can bind hCG simultaneously with hCG binding to the receptor *(5,17,18)*. Alternatively, B107 only binds intact hCG, cannot bind hCG while bound to receptor and blocks binding of free hCG to the receptor *(5,17,18)*. Monoclonal LHR29 made against the hLH/CG receptor was obtained from E. Milgrom *(13)*. Polyclonal antisera against the extracellular domain of the hLH/CG receptor was produced in our laboratory and has been previously described *(5)*.

Determination of Affinity of Fusion Protein for hCG

To determine the affinity of the fusion protein for hCG, we developed a reliable and highly reproducible technique for assaying binding using the His tag for capture of receptor-bound material. This was accomplished by using the Ni-NTA resin to capture receptor fusion protein following incubation with radiolabeled ^{125}I-hCG. Briefly, purified receptor material was incubated in 50 m*M* $NaHPO_4$/300 m*M* NaCl (pH 8.0) with ^{125}I-hCG for 2–4 h at ambient temperature or at 4°C overnight. Incubation volume was standardized at 1 mL and approx 200 ng of receptor fusion protein with 2×10^5 cpm of labeled hCG (approx 75 fmol) was added to each incubation reaction. Ni-NTA resin was prepared by extensive washing with 50 m*M* $NaHPO_4$/300 m*M* NaCl (pH 8.0) followed by resuspension in the same buffer with a volume equivalent to packed resin volume. Typically 20 μL of the resuspended resin was added to each incubation reaction, and the suspension was incubated an additional 2 h at ambient temperature with mixing every 10 min to resuspend the settled resin. The resin was then spun down and washed twice with 50 m*M* $NaHPO_4$/300 m*M* NaCl (pH 8.0) followed by the same buffer including 20 m*M* imidazole at pH 8.0. Bound hCG was identified by counting in a gamma counter. Background generated by binding of the labeled hCG alone to the resin was generally <5% of that obtained when receptor material was added to the incubation.

To facilitate an accurate determination of affinity, 2×10^5 cpm of ^{125}I-hCG was titrated with decreasing amounts of receptor material until the number of bound counts began to decrease. At that point, the amount of receptor material was not in excess and was just sufficient to bind the maximum number of counts (i.e., as many counts as a saturat-

ing amount of receptor material). Using this same amount of receptor material (approx 200 ng) with 2×10^5 cpm of ^{125}I-hCG (approx 75 fmol) in each incubation reaction, the affinity of the expressed receptor fusion for hCG was determined by adding decreasing amounts of cold hCG to each incubation reaction such that the concentration of cold hCG varied between 10^{-7} and 10^{-12} *M*. The data were plotted as molarity of cold hCG vs percentage of maximally bound ^{125}I-hCG (Fig. 3). Maximally bound ^{125}I-hCG is equal to the number of counts bound in the absence of cold hCG. The percentage of maximally bound ^{125}I-hCG is then determined from the following equation: (cpm of bound hCG in presence of test amount of cold hCG)/(cpm of maximally bound hCG) $\times$ 100. The percentage of maximally bound ^{125}I-hCG is presented instead of absolute counts so that data could be readily compared from a series of binding experiments that may have employed different preparations of labeled hCG and receptor protein. Graphs were plotted with the program Axum 5.0 (Mathsoft, Cambridge, MA), and a best-fit curve was drawn through the data points using the Lowess algorithm.

Effect of MAbs B105 and B107 on hCG Binding to Receptor

To test the effect of B105 and B107 on binding of labeled hCG to the receptor, we titrated various dilutions of these antibodies with labeled hCG, in the binding assay described in the preceding section, to determine an appropriate antibody dilution for these studies. High concentrations of a given MAb will simulate binding since MAbs bind the Ni-NTA resin nonspecifically and therefore bring down labeled hCG in the resin pellet. Therefore, we chose as a working dilution of antibody a concentration that caused an increase of 10% or less in the background binding of hCG to the Ni-NTA resin (i.e., labeled hCG and resin). Appropriate dilutions of B105 and B107 were incubated with ^{125}I-hCG and the fusion protein in the binding assay. Addition of these dilutions of antibodies in the Ni-NTA binding assay had the desired effect of blocking binding to recombinant receptor in the case of B107 and having little or no effect in the case of B105 (Fig. 4).

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References

1. Ingley, E. and Hemmings, B. A. (1999). *Protein Exp. Purif.* **17,** 224–230.
2. Qiu, J., Swartz, J. R., and Georgiou, G. (1998). *Appl. Environ. Microbiol.* **64,** 4891–4896.
3. Cheadle, C., Ivashchenko, Y., South, V., Searfoss, G. H., French, S., Howk, R., Ricca, G. A., and Jaye, M. (1994). *J. Biol. Chem.* **269,** 24,034–24,039.
4. Huth, J. R., Norton, S. E., Lockridge, O., Shikone, T., Hsueh, A. J., and Ruddon, R. W. (1994). *Endocrinology* **135,** 911–918.
5. Lobel, L. I., Rausch, P., Trakht, I., Pollak, S., and Lustbader, J. W. (1997). *Endocrinology* **138,** 1232–1239.
6. di Marzo, V., Willis, A. E., Boyer-Thompson, C., Appella, E., and Perham, R. N. (1994). *J. Mol. Biol.* **243,** 167–172.
7. Greenwood, J., Willis, A. E., and Perham, R. N. (1991). *J. Mol. Biol.* **220,** 821–827.
8. Chen, W. and Bahl, O. P. (1993). *Mol. Cell. Endocrinol.* **91,** 35–41.
9. Osuga, Y., Kudo, M., Kaipia, A., Kobilka, B., and Hsueh, A. J. (1997). *Mol. Endocrinol.* **11,** 1659–1668.
10. Prinz, W. A., Aslund, F., Holmgren, A., and Beckwith, J. (1997). *J. Biol. Chem.* **272,** 15,661–15,667.
11. Stewart, E. J., Aslund, F., and Beckwith, J. (1998). *EMBO J.* **17,** 5543–5550.
12. Wimalasena, J., Dostal, R., and Meehan, D. (1992). *Gynecol. Oncol.* **92,** 345–350.
13. Meduri, G., Charnaux, N., Loosfelt, H., Jolivet, A., Spyratos, F., Brailly, S., and Milgrom, E. (1997). *Cancer Res.* **57,** 857–864.
14. Lobel, L., Pollak, S., Wang, S., Chaney, M., and Lustbader, J. W. (1999). *Endocrine* **10,** 261–270.
15. Dufau, M. L. and Kusuda, S. (1987). *J Recept. Res.* **7,** 167–193.
16. Dufau, M. L. (1998). *Annu. Rev. Physiol.* **60,** 461–496.
17. Lustbader, J. W., Yarmush, D. L., Birken, S., Puett, D., and Canfield, R. E. (1993). *Endocr. Rev.* **14,** 291–311.
18. O'Connor, J. F., Birken, S., Lustbader, J. W., Krichevsky, A., Chen, Y., and Canfield, R. E. (1994). *Endocr. Rev.* **15,** 650–683.
19. Cole, L. A., Shahabi, S., Oz, U. A., Bahado–Singh, R. O., and Mahoney, M. J. (1999). *Clin. Chem.* **45,** 2109–2119.
20. Birken, S., Chen, Y., Gawinowicz, M. A., Lustbader, J. W., Pollak, S., Agosto, G., Buck, R., and O'Connor, J. (1993). *Endocrinology* **133,** 1390–1397.
21. Birken, S., Gawinowicz, M. A., Kardana, A., and Cole, L. A. (1991). *Endocrinology* **129,** 1551–1558.
22. Laemmli, U. K. and Favre, M. (1973). *J. Mol. Biol.* **73,** 575–599.
23. Laemmli, U. K. (1970). *Nature* **70,** 680–685.
24. Morrissey, J. H. (1981). *Anal. Biochem.* **117,** 307–310.
25. Towbin, H., Staehelin, T., and Gordon, J. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
26. Burnette, W. N. (1981). *Anal. Biochem.* **112,** 195–203.