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Purification of the Growth Hormone Releasing Hormone Receptor with a C-Terminal, Biotinylated Affinity Ligand

John R. Zysk,[1] Bruce D. Gaylinn,* Charles E. Lyons,* Bonita Johnson, C. Mark Eppler, William R. Baumbach, and Michael O. Thorner*

*Agricultural Research Division, American Cyanamid Company, Princeton, New Jersey 08543; and *Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908*

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The receptor for growth hormone-releasing hormone (GHRH) has been purified from bovine pituitary tissue and HEK293 cells transfected with human or porcine receptor using a retrievable biotinylated GHRH analog. Custom synthesized [His[1], Nle[27], Biotin-Lys[41]]-human GHRH-(1–41)-NH_2 ($GHRH_b$) bound to pituitary membranes with affinity comparable to human GHRH. $GHRH_b$ which has the biotinyl group on the C-terminus of the peptide allowed simultaneous binding to both the receptor and streptavidin agarose. This analog was used directly in the purification of the receptor from pituitary tissue or was modified by incorporation of the photoaffinity group ANBNOS ($GHRH_{\lambda b}$), radioiodinated and used to demonstrate purification of the GHRH receptor from transfected HEK293 cell membranes. Membranes were prepared and prebound with the respective ligand followed by CHAPS-solubilization and application of the solubilized complex to a strepavidin agarose column. Analysis of eluates from the pituitary tissue purification by silver stained SDS PAGE or of autoradiographs of gels from HEK293 eluates revealed specific bands of 52 and 55 kDa, respectively. The higher size of the latter band is expected for the ligand-crosslinked receptor. Both bands displayed similar mobility shifts of 10 kDa upon treatment with N-glycosidase, a method previously used to characterize this receptor (1). A 45 kDa band corresponding to the size of the $G_{S\alpha}$ subunit was also detected in eluates of the silver stained gels, suggesting that the GHRH receptor was retrieved as a heterotrimeric complex. Fold purification and yield for this procedure were estimated to be greater than 50,000 and 2.6–9%, respectively.

The receptor for growth hormone releasing hormone (GHRH) represents a key element in the control of growth hormone (GH) secretion in the pituitary somatotroph. The GHRH receptor exhibits the hallmarks of a G-protein linked receptor (1–4) and has been shown to have the consensus sequence for the nonneurokinin receptor family which includes the secretin and VIP receptors (5–7). Despite these characterizations, a suitable methodology for purification of this receptor has not been described. The primary reasons for this are low receptor expression in pituitary tissue (8,9), high background binding by the ligand (10), and a lack of information regarding detergent solubilization of the receptor which would facilitate high affinity binding and purification by chromatographic methods. The successful use of retrievable, biotinyl-ated ligands for the purification of the somatostatin (SRIF, 11, 12), μ opioid receptors (13), and more recently the PACAP receptor (14) has prompted a similar approach for the GHRH receptor. A biotinylated ligand provides a means of prebinding the membrane-bound receptor and retrieving the high affinity complex after detergent solubilization on a solid phase support. The present communication describes the characterization of the biotinylated ligand and the purified GHRH receptor. Validation of this methodology involved the use of photoaffinity probes similar to those described previously (1) but with the addition of a biotinyl group at the C-terminus. The significance of this method derives from its simplicity, since GHRH receptor can be purified without resorting to fusion protein constructs and pure receptor can be obtained in the native (glycosylated) state.

[1] Correspondence to John Zysk, American Cyanamid Company, PO Box 400, Princeton, NJ 08543. FAX: 609 799 1842.

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MATERIALS AND METHODS

Materials. The C-terminal biotinylated GHRH analog (His[1], Nle[27], Biotin-Lys[41])human GHRH-(1–41)-NH_2 ($GHRH_b$) was custom synthesized by Anita Hong of BioServ Labs (San Jose, CA, formerly Nuros Corporation). The remaining peptides were obtained from Peninsula Laboratories (Belmont, CA) and include (His[1],Nle[27]) human GHRH-(1–32)-NH_2 ($GHRH_a$), rat GHRH and VIP. Streptavidin-agarose and the photoprobe N-5-azido-2-nitrobenzyloxysuccinimide (ANB-NOS) were purchased from Pierce (Rockford, IL). Bovine pituitary tissue was from Pelfreeze (Rogers, AR) and ovine pituitaries were from lain Clarke (Prince Henry's Inst. of Med. Res., Melbourne, Australia). Endoglycosidase-F and N-glycosidase-F were purchased from Boehringer Mannheim (Indianapolis, IN). All reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). Unless specified, all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Iodination of $GHRH_a$ and $GHRH_b$. Both analogs were iodinated to low stoichiometry on Tyr [10] and purified by reverse phase HPLC as described previously (1).

Preparation of the biotinylated GHRH photoprobe. The photoaffinity, biotinylated analog $GHRH_{\lambda b}$ was prepared by covalent coupling of ANBNOS to $GHRH_b$ followed by HPLC purification (1).

Strepavidin-coupled agarose. Strepavidin agarose (SA) was washed three times in 10× volume of GHRH/CHAPS solubilization buffer containing 50 mM Tris, pH 7.4, 2 mM EGTA, 5 mM $MgCl_2$ and 5 mM CHAPS (3-[(3-chloamidopropyl)-dimethylammonio]-1-propanesulfonate). The washed SA was then used directly in the purification protocol.

Preparation of crude pituitary membranes. Unless stated, all steps were performed at 4°C. Fifty to one hundred grams of frozen bovine pituitary tissue was ground to a powder in the presence of dry ice in a coffee mill. To the frozen tissue was added three volumes (150- 300 ml) of homogenization buffer containing 50 mM HEPES, 100 mM NaCL, 10 mM EDTA, pH 7.4 with 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 200 U/ml aprotinin. The preparation was then homogenized in a Waring blender for one minute (two, 30 second bursts) and filtered through three layers of cheesecloth. The filtered homogenate was centrifuged for five minutes at 600 × g and the supernatant was retained. The supernatant was centrifuged for 25 minutes at 20,000 × g and the pellets were retained. The pellets were resuspended in 150–300 ml of GHRH binding buffer containing 50 mM Tris, 2 mM EGTA, 5 mM $MgCl_2$, 0.05 mg/ml alamethicin, 30 μg/ml bacitracin and protease inhibitors as above. This membrane preparation was used directly in the purification protocol.

Binding. Crude pituitary membranes were incubated with radioiodinated ligand and then pelleted and counted (1) or CHAPS solubilized and counted after separation of unbound ligand with charcoal/dextran (5% Norit A/0.5% Dextran T-70, (1,15)).

Photoaffinity crosslinking and retention of complexes on strepavidin agarose. The radiolabeled, $GHRH_{\lambda b}$ photoaffinity probe was bound to pituitary and HEK293 membranes (from 20 mg pituitary per tube) as described previously (1). Pellets were solubilized in GHRH binding buffer containing 5 mM CHAPS, mixed with one tenth volume of charcoal/dextran and spun in a microcentrifuge for three minutes. Supernatants (400 μl) were incubated at 4° C with (10 μl) streptavidin agarose for three hours. The strepavidin agarose was flashed with UV light (1), washed extensively (4 × 200 μL) in solubilization buffer containing 1× phosphate buffered saline, pH7.2, no Ca^{2+} or Mg^{2+} (GIBCOBRL, Paisley, Scotland), 0.1% SDS, 1% nonidet P-40, 0.5% deoxycholate and 0.5 M NaCl and then pelleted in a microcentrifuge. The pellets were suspended in SDS sample buffer, boiled for 5 min. at 100°C and run on 10% SDS polyacrylamide gels and autoradiographed. Autoradiographes were standardized against molecular weight markers as described (1).

Purification protocol. Membranes were prepared from 120 grams of bovine pituitary tissue and diluted to a final volume of 250 ml in binding buffer. $GHRH_b$ was added to 200 ml of membranes to a final concentration of 10^{-8} M. The remaining 50 ml of membranes was incubated with 10^{-7} M nonbiotinylated GHRH. Membranes were incubated overnight at 4°C with gentle stirring or alternatively, by rotating end over end on a Labquake Rotator (Labindustries, Inc., Berkely, CA). The membranes were pelleted at 20,000 × g for 25 minutes. The pellets were resuspended in the original volume of binding buffer and recentrifuged. The resulting pellets were resuspended in GHRH/CHAPS solubilization buffer. After solubilization on ice for 90 minutes (with occasional vortexing), the mixtures were centrifuged at 100,000 × g for 35 minutes (Beckman Ti 35) and the supernatants (200 ml and 50 ml) were incubated with streptavidin agarose (1/40 the volume of the supernatant). for four hours at 4° C by end-over-end rotation. Each mixture was poured into a respective column and washed extensively in the following manner: 20 column volumes GHRH/CHAPS, 20 column volumes GHRH/CHAPS containing 0.5 M NaCl, four column volumes GHRH/CHAPS. The columns were eluted with GHRH/CHAPS, pH 5. The first four column volumes of the eluate were combined and concentrated on a Centricon 30 to approximately 100 μl. This concentrate was extracted (16) and analyzed on silver stained, SDS polyacrylamide gels (17).

Iodination and deglycosylation of a purified protein separated as a 52 kDa band by SDS polyacrylamide gel electrophoresis. Samples of the pH5 elution which had been solubilized in SDS sample buffer were extracted in chloroform/methanol and radioiodinated by the chloramine T method (18). The iodinated products were separated on Sephadex G-25-300 in PBS + 0.1%SDS and 1% ovalbumen. Part of the radioiodinated fraction was reconstituted in GHRH binding buffer and treated with a combination of endoglycosidase-F and N-glycosidase-F (N-glycosidase treatment (1)). Samples were then solubilized in SDS sample buffer and separated by SDS polyacrylamide gel electrophoresis. Gels were dried and autoradiographed for analysis.

RESULTS

Comparative binding of GHRH in bovine and ovine pituitaries. Due to the limited amount of ovine pituitary available, purification of the receptor was performed in bovine pituitary tissue. GHRH specifically binds with high affinity to membranes from ovine pituitary tissue (1) and exhibits similar crosslinking patterns and specificity with bovine pituitary, the latter exhibiting a slightly higher M_r (Figure 1). Moreover the shift in size of the crosslinked bovine receptor after deglycosylation by N-glycosidase was proportional to that of the ovine counterpart (Figure 2).

Binding of ^{125}I-GHRH$_b$ and ^{125}I-GHRH$_a$ to streptavidin-agarose beads. Incubation of radiolabeled $GHRH_b$ with streptavidin-agarose beads for 30 minutes at room temperature resulted in the retention of 83% of the label initially bound to the beads after a single washing in binding buffer. Labeled $GHRH_a$ however resulted in the retention of less than 30% of the ligand after the same treatment. This 30% background could be further reduced with more stringent washes.

Formation of receptor-streptavidin complexes with ^{125}I-GHRH$_{\lambda b}$. Membranes form HEK293 cells transfected with the porcine GHRH receptor were labeled with the biotinylated or nonbiotinylated photoaffinity probe, solubilized in GHRH/CHAPS buffer and incubated for three hours with streptavidin agarose at 4°C followed by washing with the same buffer. The washed beads were UV flashed for 15 minutes and then boiled in SDS sample buffer for 5 minutes and applied to SDS polyacrylamide gels. Although silver stained gels showed no trace of protein bands, autoradiographs of the gels revealed a 55 kDa band in beads incubated with $GHRH_{\lambda b}$-labeled membranes which could be displaced by 10 nM $GHRH_a$. (Figure 3). Similar results were also obtained using membranes from ovine pituitaries (data not shown). These data establish that the biotinylated GHRH ligand could form a soluble complex between streptavidin sepharose and the receptor.

Purification of the soluble GHRH receptor by streptavidin-agarose chromatography. Prebinding of pituitary membranes with GHRH followed by solubilization in CHAPS results in a very stable receptor/ligand complex (Figure 4A- bar graph). Significant dissociation of this complex was possible at pH 5 (Figure 4B). Purification of the solubilized complex on streptavidin-agarose was performed by elution of the receptor at pH 5 after extensive washing of the column. Eluate from this step resulted in three major bands by silver-stained SDS polyacrylamide gels (Figure 5, lane 1). The largest molecular weight band (70 kDa) was determined to be nonspecific since it also was apparent in the control column (Figure 5, lane 2) in which no biotinylated ligand was used. Two other bands (52 and 45 kDa) were only visible in the column in which biotinylated ligand was used (Figure 5, lane 1). The 52 kDa band corresponds closely to the size of the photoaffinity labeled receptor. This pattern was obtained in five separate runs. Radioiodination of the pH 5 eluate and treatment with endoglycosidase F resulted in a shift in the size of the band to 42 kDa. This shift

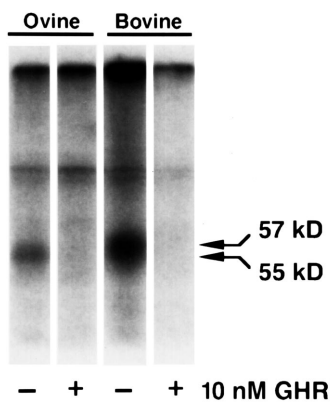

FIG. 1. Autoradiograph of photoaffinity crosslinked GHRH receptors from ovine and bovine pituitary tissue. Crude membranes were photocrosslinked, CHAPS solubilized and separated by SDS PAGE as described in the Methods.

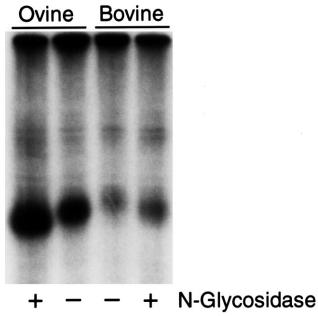

FIG. 2. Treatment of photoaffinity crosslinked ovine and bovine GHRH receptors with N-glycosidase. CHAPS solubilized membranes were treated with and N-glycosidase as previously described (1).

is proportional to that observed when the photoaffinity-labeled GHRH receptor was treated with endoglycosidase F (Figure 6). The 45 kDa band was present only in the presence of biotinylated ligand and corresponds to the size of the alpha subunit of the stimulatory G-protein. Fold purification and yield based on results from the photoaffinity purifications using cpm of ^{125}I-GHRH$_{\lambda b}$ retained on streptavidin columns and analyzed on SDS polyacrylamide gels indicate approximately 50,000 fold purity and 2.6–9.9% yield (crude membranes = 100%), respectively.

DISCUSSION

Many G-protein coupled, peptide hormone receptors have been purified by a common strategy involving detergent solubilization of plasma membranes followed by ligand affinity chromatography. For some receptors, including GHRH, binding after solubilization is not optimal and may be of low affinity. A possible reason for this is that solubilization disrupts G-protein-receptor coupling. The strategy used in this report is based on an approach which was used to successfully purify the somatostatin (11) and the μ opioid receptors (17) and involves prebinding a bifunctional affinity ligand with plasma membranes *followed by* solubilization and retrieval of the soluble receptor-ligand complex by affinity chromatography. This method does not involve constraints on ligand binding to the receptor provided the biotinyl group does not interfere with the binding domain. Moreover, if the biotinyl group is removed from the bound portion of the ligand by a spacer of appropriate length, it should be possible to bind the soluble complex to streptavidin in solid phase. Initially, GHRH biotinylated at lys^{12} or lys^{21} was tested and found to bind the receptor, but not in the presence of streptavidin (data not shown). This is most likely a steric hindrance problem due to the large size of streptavidin and led to the design of GHRH$_b$ with the biotin at position 41 which poses no such problem.

A major advantage of the purification method used for the GHRH receptor in this report is the

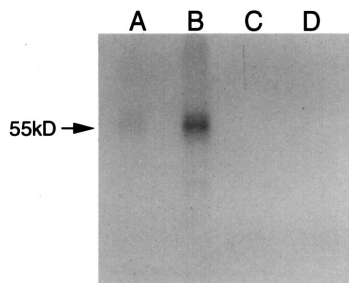

FIG. 3. Autoradiograph of SDS-eluted, photoaffinity (GHRH$_{\lambda b}$)-labeled, porcine GHRH receptor from streptavidin agarose. Column A: pre-SDS wash. Column B: SDS eluate. Columns C and D are respectively identical to columns A and B except that they contained 10^{-7} M GHRH$_a$ in the initial binding step.

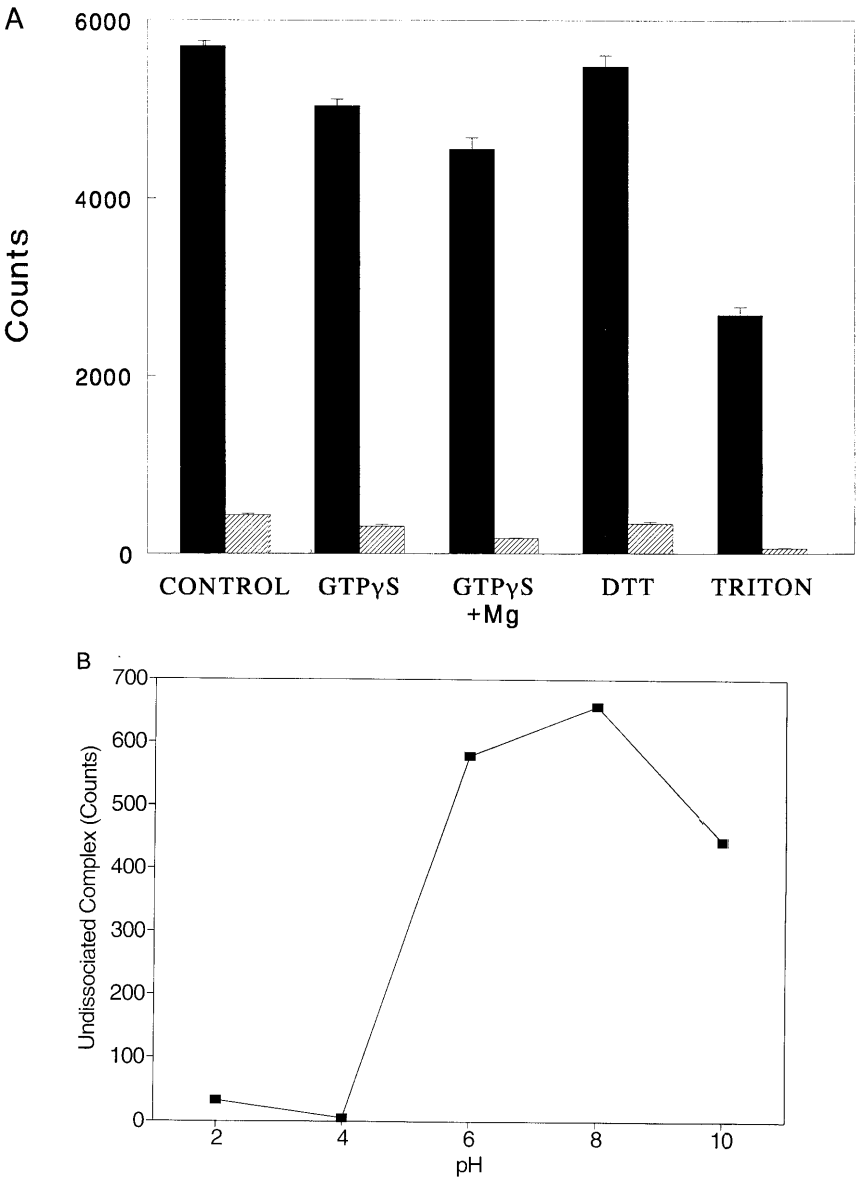

FIG. 4. Stability of CHAPS solubilized GHRH receptor:ligand complex. A. Stability of the receptor form ovine tissue in the presence of various factors. Binding of ^{125}I-GHRH$_a$ was measured in the absence (solid bars) or presence (cross-hatched bars) of 10^{-8} M unlabeled GHRH$_a$ B. pH profile of the solubilized receptor:ligand complex.

remarkable stability of the streptavidin-GHRH$_b$-receptor complex (Figure 4A). This stability has allowed extensive washing of the solid phase with the resultant high purity of the receptor protein. The effectiveness of this procedure is especially significant when the high nonspecific binding properties of the GHRH peptide are considered (1,7). Presumably, nonspecific binding of GHRH is due to the negatively charged nature of the peptide and CHAPS solubilization favors the high affinity complex (1). The susceptability of the solubilized GHRH$_b$-receptor complex to dissociation by acidic pH (Figure 4B) was used to advantage as an elution step after nonspecific proteins were washed from the streptavidin agarose. As shown in Figure 5, only one major nonspecific band

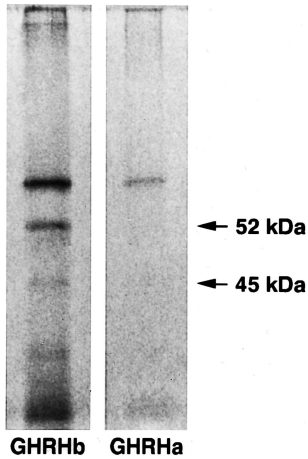

FIG. 5. Silver-stained SDS polyacrylamide gel of the purified bovine GHRH receptor.

(~70kDa) was visible by silver stain in pH 5 eluates from the affinity column and the control column.

Verification of the 52 kDa band as the receptor was ascertained by specificity of elution from the affinity column *vis a vis* the absence of this band in eluates from the control column, and by deglycosylation of this band by N-glycosidase (Figure 6). Deglycosylation of this band resulted in a decrease in molecular weight exactly as predicted from the deglycosylation of the photoaffinity labeled receptor. The photoaffinity labeled receptor decreased in size from 55 to 45 kDa in ovine pituitary and 57–47 in bovine as apposed to the purified (bovine) band which shifted from 52 to 42 kDa. The difference in size is accounted for by the presence of the ligand in the photoaffinity labeled band.

Of further significance is the presence of a 45 kDa band found only in the affinity column eluate. This band corresponds in size to the alpha subunit of the stimulatory G-protein G_s. Although this protein is a cholera toxin substrate for ADP-ribosylation, attempts at labeling this band with ^{32}P-ADP were unsuccessful, presumably because of the lack of ADP ribosylation factor (ARF), a needed cofactor in this process (19). However, the alpha subunit of G_i was copurified from the somatostatin receptor using the same purification approach (11) and it is assumed that the receptor/

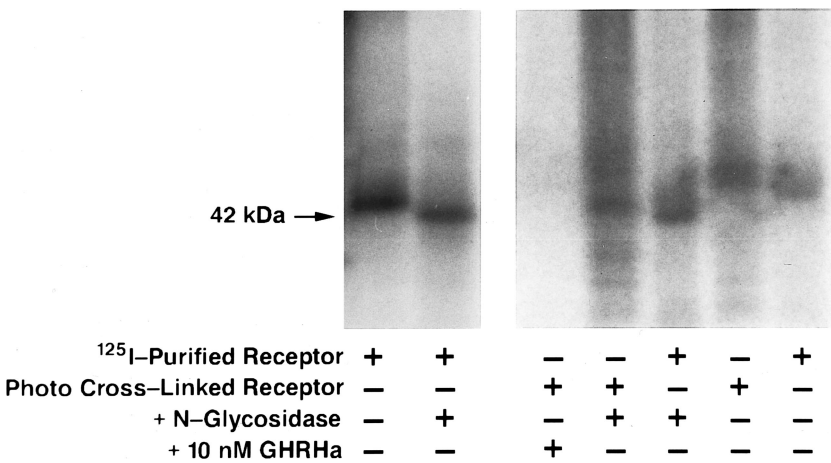

FIG. 6. Deglycosylation of the 52 kDa bovine GHRH receptor following affinity purification.

G-protein complex are copurified. It was possible to elute the somatostatin receptor as well as the receptors for the μ opiod receptor (13) and the adenosine receptor (20) with GTPγS. Unfortunately, the *solubilized* GHRH receptor-ligand complex is resistant to dissociation by guanine nucleotides (Figure 4A) although the nonsolubilized receptor is GTP sensitive (1,4).

Based on Scatchard analysis of crude membranes from pituitary tissue and the intensity of the 52 kDa band on silver stained gels we have estimated that the amount of receptor purified per gram of tissue to be approximately 0.5–3 ng ($\sim$10–60 fmoles). From these estimates the relative yield for this receptor is approximately 5% (range 2.6– 9). Fold purification starting from crude membranes is estimated to be greater than 50,000. The effectiveness of the purification procedure may be greatly enhanced with clonal cell lines as starting material since the amount of receptor in normal pituitary tissue is so small and purification from individual batches of tissue inconsistent. An estimate for the yield of porcine GHRH receptor in transfected HEK293 cells from the *solubilized* receptor stage to elution from the SA affinity matrix was 17%. Based on the estimated number of binding sites in this cell line (21) the overall yield from whole cells would be approximately 5%. The method will also be useful in the isolation of the native form of the receptor from pituitary cell lines, eliminating the necessity for using fusion protein constructs as aids in purification. This may be particularly relevant in studies involving the endogenous glycosylation of the receptor or in the retrieval of the intact receptor for analysis of site-specific phosphorylation or structure-function studies where modification of the parent protein may not be desirable.

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