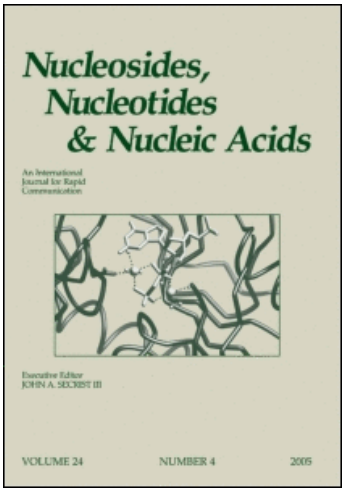

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Joachim W. Engels[a]; Jan Glauder[a]; Hubert Mülliner; Eugen Uhlmann; Waldemar Wetekam; T. Hashimoto Gotoh; Barbara Scheikl-lenz[a]
[a] Universität Frankfurt, Institut für Organische Chemie und Biologische Chemie, Frankfurt/Main

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CHEMOENZYMATIC GENE SYNTHESIS OF A GENE FOR HUMAN GROWTH HORMONE RELEASING HORMONE (hGHRH), ITS EXPRESSION IN E. COLI AND ENZYMATIC AMIDATION

Joachim W. Engels,* Jan Glauder, Hubert Müllner†, Eugen Uhlmann†, Waldemar Wetekam†, T. Hashimoto Gotoh† and Barbara Scheikl-Lenz

Universität Frankfurt, Institut für Organische Chemie und Biologische Chemie, D-6000 Frankfurt/Main 50
†Hoechst AG, P.O.box 800320, D-6230 Frankfurt/Main 80

Abstract

A gene coding for hGHRH was constructed from chemically synthesized oligonucleotides in two fragments; After subcloning and joining the total gene was expressed in E. coli as a fusion protein with ß-galactosidase. Chemical cleavage thereof with cyanogen bromide, purification of Leu^{27}GHRH-Gly^{45} by chromatography followed by enzymatic conversion yielded Leu^{27} GHRH-NH_2.

INTRODUCTION

Recently two independant studies characterized the human growth hormone releasing hormone (hGHRH) from a pancreatic islet tumor as 44- or 40-amino acids containing peptides[1,2] by sequencing techniques. The natural hormone occurs in the C-terminal amidated form, which in vitro and in vivo shows the higher activity than the corresponding acid. The authors proved their structure assignments by peptide synthesis and could in addition show the release of growth hormone in vitro and in vivo[3,4]. Co- or posttranslationally modified peptides and proteins pose a problem for the expression of synthetic genes in E. coli. This organism lacks the necessary enzymatic machinery in order to alter the peptides after protein synthesis. Thus our approach

constructed the gene for GHRH in such a way as to express the peptide in a preform which will be recognized and modified to the amide by an amidation enzyme[5]. The enzyme a peptidyl- α -amidating monooxygenase (PAM) was shown to be present in several mammals, notably pigs[6]. This enzyme catalyses the transformation of a C-terminal glycine to a glyoxylic acid, leaving the amide nitrogen on the preceeding amino acid. (FIG.1)

RESULTS AND DISCUSSION

Gene synthesis

According to the published peptide sequence[1] we constructed a synthetic gene[7] based on the genetic code. We selected the codons of highly expressed proteins in E. coli[8] avoiding also unfavourable palindromic sequences and possible stable secondary structures on the mRNA-level. The total gene, 149 base pairs in length was divided into two subfragments GHRH I and GHRH II (FIG. 2). A total of 13 oligonucleotides ranging in length from 16 to 28 nucleotides were synthesized by solid phase phosphoramidite chemistry[9] on a fractosil 500-support. After final deprotection by concentrated ammonia the crude oligonucleotides were applied directly to a 14% polyacrylamide preparative gel and separated by electrophoresis. Detection by uv shadowing at 254 nm, cutting out the dominant bands and extraction from the gels by water, followed by desalting on Sephadex G50 and Biogel P6 furnished the appropriate oligonucleotides Ia - If and IIa - IIg.

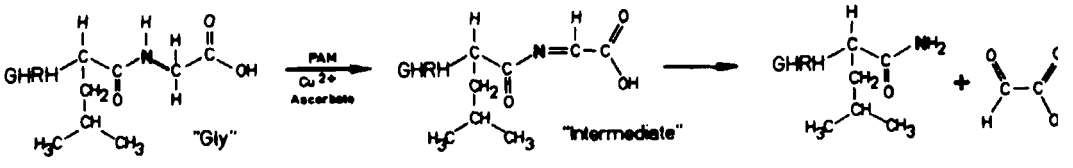

FIG. 1. Proposed-mechanism for PAM Amidation

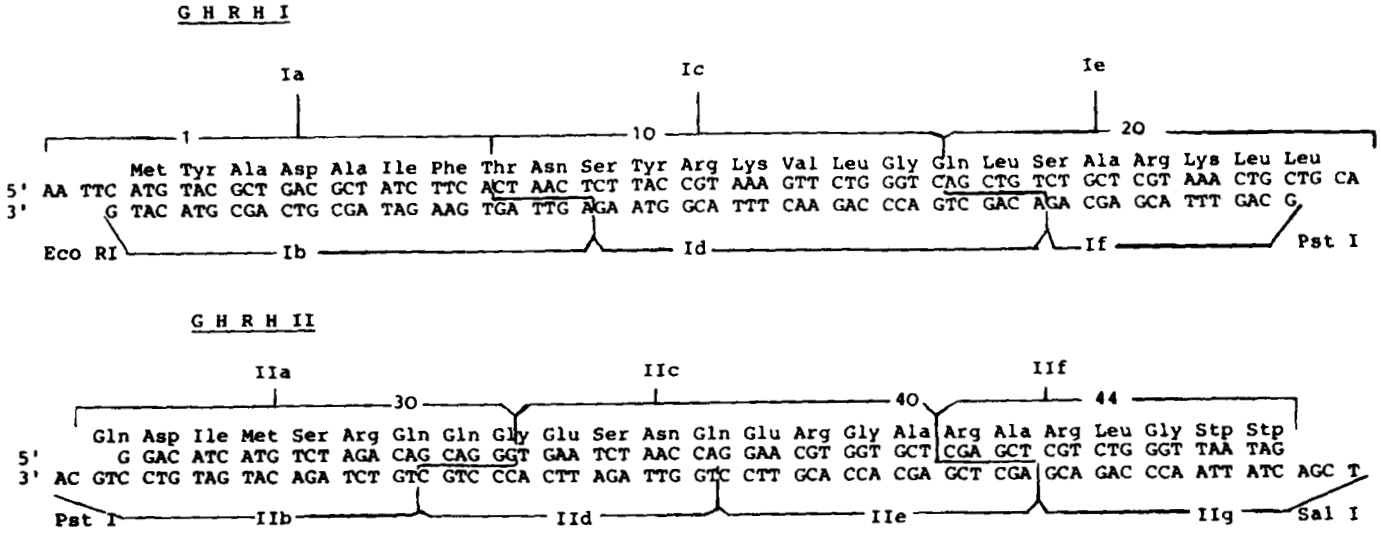

FIG. 2. Nucleotide and amino acid sequence of the synthetic human GHRH-Gly^{45}-gene. Individual oligonucleotides (Ia-If, IIa-IIg) are arrowed. Numbers above each line refer to amino acid position.

Their purity and homogeneity was checked by kinasing the oligonucleotides with ^{32}P-ATP and running analytical gels. Alternatively the oligonucleotides were purified by hplc ion exchange chromatography using triethylammoniumphosphate buffers in combination with acetonitrile (25% in water) as organic phase. Subsequently for enzymatic gene assembly 1 nmole of each deoxyoligonucleotide was denatured by heating at 90°C and quick chilling on ice, then phosphorylated at the 5' end with T-4 polynucleotide kinase[10] except the segments Ia, If, IIa, and IIg in 20 µl 50 mM Tris-HCl buffer pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 5 nmol ATP and incubated with 5 units T4 kinase for 1/2 h at 37°C. The enzyme was denatured at 95°C. For ligation the pieces Ia - If were hybridized in pairs taking 1.2 nmoles of Ia and If and 1 nmole of Ib - Ie in 50µl at 90°C for 2 min. and slowly cooling to room temperature (1 h). The fragments Ia - If were pooled and ligated in 50 mM Tris-HCl buffer pH 7.6, 20 mM $MgCl_2$, 10 mM DTT with 100 units T-4 ligase at 15°C within 16 h. The same reaction could later be obtained by directly annealing Ia - If and IIa - IIg and adding ligase in the same way as described. The reaction mixture was directly applied to a 10% polyacrylamide gel and the bands were visualized by ethidium bromide staining. The DNA-duplexes GHRH I and GHRH II were isolated from the intermediates and starting materials.
Elution from the gel and desalting on sephadex G50 gave the duplexes GHRH I and GHRH II in 50 pmole quantities which after kination were ready for cloning in the pUC plasmids.

Cloning of synthetic hGHRH I and hGHRH II DNA fragments

The synthetic DNA fragment GHRH I comprising the amino-terminal half of GHRH was cloned into the Eco RI/Pst I site of plasmid pUC 8 [11], FIG. 3. Individual clones were characterized by restriction enzyme analysis and subsequent sequencing. In a similar approach, the DNA fragment GHRH II comprising the distal half of the molecule was cloned into the Pst I/Sal I site of pUC 9 and the correct sequence

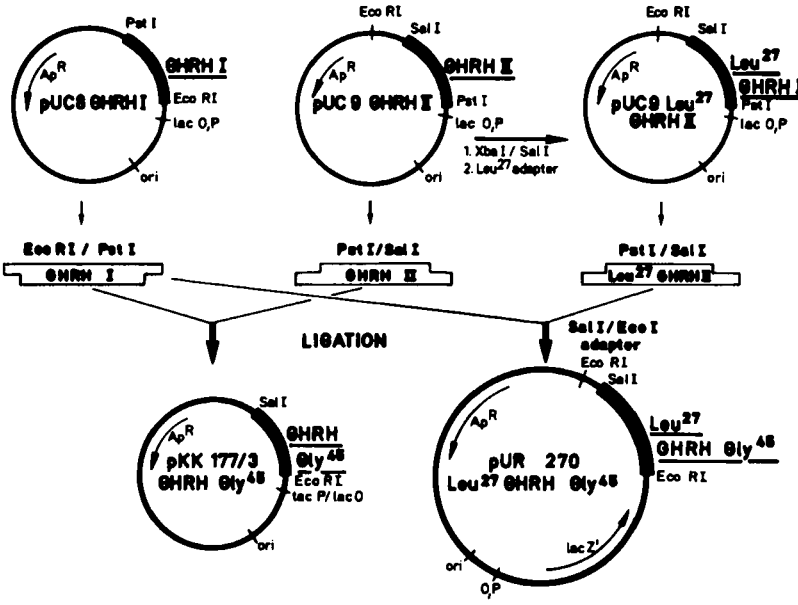

FIG. 3. Construction of the two expression plasmids pkk 177/3-GHRH-Gly and pUR 270 Leu^{27}GHRH-Gly^{45}, amp^{r}, ampicillin resistance.

determined. Both fragments GHRH I and GHRH II were isolated from the appropriate clones by Eco RI/Pst I fragmentation of DNA from clone GHRH I and by Eco RI/Pst I from clone GHRH II. After ligation of equimolar amounts of both fragments (50 ng each) the reaction product was ethanol precipitated and recut by the restriction enzyme Eco RI. Gel electrophoresis of the restricted ligation products yielded a predominant band of 149 base pairs. This DNA fragment comprising the information of Met^{27} hGHRH-Gly^{45}-OH was subsequently introduced into a direct expression plasmid pKK 177/3[12] (FIG. 3). Plasmid pUC 9[11] (FIG. 3) was cleaved by Xba I/Eco RI and this fragment was isolated on 10% polyacrylamide gels. This fragment was incubated in a ligation reaction with a synthetic adapter top (5'GGACATCCTGT) bottom (5'CTAGACAGGATGTCCTGCA) in 10 fold molar excess.

This mutagenic adapter was designed such that the methionine codon ist replaced by a one base pair exchange and converted to a leucine codon based on a secondary structure prediction[13]. The ligation reaction product was subsequently ethanol precipitated, cleaved by the restriction enzymes Eco RI/Pst I and separated on a 10% polyacrylamide gel. The appropriate DNA band was eluted from the gel and ligated into plasmid pUC 9 as described above. The resulting recombinat plasmid contains now GHRH II (leucine 27 codon) fragment, which can be used for further cloning in fusion expression plasmids as described in FIG. 3.

Direct expression-construction for GHRH

One mode of expression of GHRH in E. coli using the unmodified GHRH gene to yield Met^{27}-hGHRH-Gly^{45}-OH without any NH_2-terminal extensions. We chose the plasmid pKK 177/3[12] with a strong tac promotor, inducible by IPTG, with its own ribosomal binding site and a convenient polylinker (pUC 9) downstream from the ribosomal binding site. This enabled us to clone the ligated GHRH into the Eco RI/Sal I site of plasmid pKK 177/3 separating the AUG-initiator codon from the ribosomal binding site by 10 nucleotides. Restriction enzyme analysis and subsequent sequencing data revealed the correct insertion of gene GHRH into plasmid pKK 177/3. In this direct expression system, using the tac regulation region the yield of immunoreactive Met^{27}-GHRH-Gly^{45} was extremely low. After 45 min. of induction by IPTG the level of accumulated Met^{27}-GHRH-Gly^{45} amounts to 50 µg per liter of cell culture. Cells (W3110 wild type) were grown in standard tryptone yeast broth to an optical density of 1 at a wavelength of 578 nm. After induction with IPTG, cells continued to grow for 45 min., were chilled immediately in ice, centrifuged down and stopped by the addition of chloroform and 0.1N HCl. After sonication, the cell debris was spun down and the lysate supernatant neutralised with Tris base to pH 7.0. It was finally tested for the presence of GHRH in a standard radioimmunoassay, using chemically synthesized GHRH (1-44)-OH as tracer. With-

out the addition of chloroform/HCl the amount of residual Met^{27}-GHRH-Gly^{45} in the lysate was less than 50 ng/liter of culture. This low yield could clearly be attributed to the rapid degradation of newly formed Met^{27}-GHRH-Gly^{45} in the cells. Induction periods longer than 45 min. as well as expression in a mutant host D29B$_1$, harbouring a peptidase mutation, which stabilized the ß-galactosidase-α-fragment in E. coli did not increase the yield of Met^{27}-GHRH-Gly^{45}.

Fusion expression construction

As an alternative approach to the direct expression of hGHRH in E. coli we chose the fusion expression with ß-galactosidase. This is believed to increase the stability of the newly formed hGHRH polypeptide in a chimeric form with 1006 amino acids of ß-galactosidase. In order to cleave the intact GHRH molecule out of a fusion product, the altered GHRH gene with a leucine codon in position 27 had to be integrated into the Eco RI site of the ß-galactosidase gene. The gene for Leu^{27}-hGHRH-Gly^{45} was integrated into the Eco RI site of plasmid pUR 270[14] by means of an Eco RI/Sal I adapter (FIG. 3). This plasmid carries the information for the ß-galactosidase up to the singular Eco RI site at amino acid position 1006 and its natural regulatory region. ß-galactosidase synthesis is induced by IPTG, and the plasmid carries, as a selectable marker, the ampicillin resistance gene. The expected fusion-protein consists of the ß-galactosidase moiety of 1006 amino acids and 45 amino acids of Leu^{27}-GHRH-Gly^{45}. Correct insertion of the GHRH into the Eco RI site of ß-galactosidase was verified by restriction enzyme analysis and sequencing of the integration site. Cultures of E. coli W3110, harbouring the ß-galactosidase-GHRH plasmid were grown in standard tryptone yeast medium to an OD_{578} of 1.0 and induced for ß-galactosidase synthesis with 0.5 mM IPTG. After 2 hours of induction, 0.5 ml of cells were withdrawn, centrifuged and lysed, taken up in 0.2 m Tris base, 0.2 M DTT, 2% SDS, 7 M urea and 0.05% bromophenol blue and loaded on a polyacrylamide gel according to Laemmli[15]. A remarkable portion of the total cell

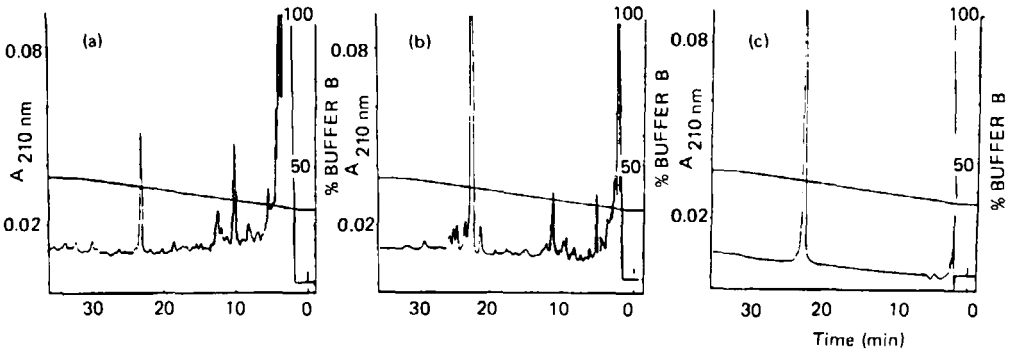

FIG. 4. Purification of Leu^{27} GHRH-Gly^{45}
A. Analytical hplc-profile of the peptides extracted from the cyanogen bromide cleavage product
B. Analytical hplc-profile of the GHRH activity containing fraction after ion exchange chromatography of the extract
C. Analytical hplc-profile of Leu^{27} GHRH-Gly^{45} after semipreparative purification by hplc Running conditions: Bio-Rad Hipore C 18 RP column, 0.1% TFA/80% acetonitrile, 0.1% TFA, H_2O; gradient from 35% to 45% eluent B in 30 min.

protein consitutes the ß-galactosidase-GHRH fusion peptide which indicates an extreme stability in the growing cell.

Isolation and purification of the Leu^{27} GHRH-Gly^{45} molecule

After induction the cells were centrifuged and lysed by decompression shock via a French press. Centrifugation of this homogenate leads to a pellet, which contains almost all of the insoluble inclusion bodies, consisting mainly of fusion protein. Threefold washing with phosphate buffer pH 7.0 (50 mM phosphate, 10 mM ß-mercaptoethanol) removes most of the containing bacterial proteins and nucleic acids. The purity of this product is roughly 30%. This partly purified fusion protein was cleaved with cyanogen bromide[16] in 60% formic acid at 30-40 °C in a closed vessel under vigorous stirring. After approximately one hour, the reaction mixture was diluted tenfold in icecold water, lyophilized and extracted with an aqueous mixture of ethanol and acetic acid (see FIG. 4).

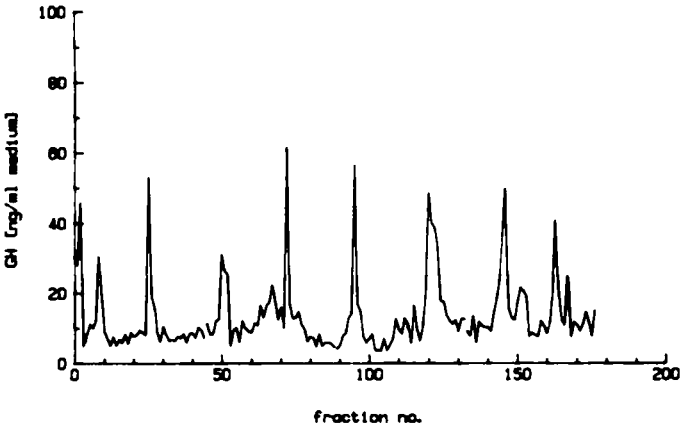

FIG. 5. Biological activity of $Leu^{27}GHRH\text{-}Gly^{45}$ in a rat pituitary cell perifusion system. Alternate pulse stimulation (5 min.) with GHRH (1-40)-OH first pulse and $Leu^{27}GHRH\text{-}Gly^{45}$.

By the first extraction more than 90% of the extractable Leu^{27}-GHRH-Gly^{45} is removed from uncleaved fragments and ß-galactosidase cleavage products. The extract (see analytical hplc profile FIG. 4A) was further chromatographed on a CM Trisacryl ion exchange column using a gradient from 100 mM NH_4Ac pH 5.0 to 500 mM NH_4Ac pH 6.0. The GHRH containing peak (analyt. HPLC profile FIG. 4B) eluted at 230 to 250 mM NH_4Ac. This fraction contains around 10 to 20% peptide. Final purification to homogeneity was accomplished by semi-preparative HPLC[17] using a 2 cm ID x 25 cm column with a 5 cm precolumn custom filled with 15 - 17 particles of Vydac Widepore RP 18 material. The peptide obtained (see FIG. 4C) represents the Leu^{27} GHRH Gly^{45} which is proven by amino acid analysis, sequence analysis and fragmentation by V8-protease (data not shown). Isoelectric focusing in ultra-thin polyacrylamide gels confirmed the similarity in charge with three other GHRH peptides. Starting with 1 kg of en-

riched fusion-protein (30% pure) the overall yield is around 3 g of highly purified peptide. This represents 33% of the theoretically possible yield.

In the biological assay, release of growth hormone (GH) in rats, Leu^{27} GHRH-Gly^{45} shows comparable potency to GHRH(1-40)-OH (Bachem) FIG. 5.

PAM-Amidation

The Leu^{27} GHRH-Gly^{45} purified as described above was then incubated with PAM. This enzyme was isolated from fresh bovine pituitaries according to a literature procedure[18]. After percoll gradient centrifugation the fractions were assayed for enzyme activity with a synthetic tripeptide dansyl-D-Ala-Pro-Gly. The most active fraction were used in the assay buffer 100 mM TES, pH 7.4, 200µg/ml catalase, 6 mM ascorbate, 6M $CuSO_4$ to transform Leu^{27} GHRH-Gly^{45} at 37°C for 3h. After stopping with 1% TFA and centrifugation the supernatant was used for hplc analysis. A new more hydrophilic product appeared on a RP 18 column, which was isolated and sequenced. The occurrence of Leu-NH_2 as the C-terminal amino acid could be ascertained by protein micro sequencing[19].

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