

SYNTHESIS AND IMMUNOLOGICAL EVALUATION OF AN EICOSAPEPTIDE RELATED TO THE C-TERMINUS OF THE β -SUBUNIT OF HUMAN CHORIONIC GONADOTROPIN

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1. Introduction

Human chorionic gonadotropin (HCG) is a glycoprotein hormone which is composed of two dissimilar subunits. The α -subunit seems to occur in closely similar form in a number of glycoproteins including LH, FSH and TSH, whereas the β -subunit contains homologous as well as specific structural regions. Sequence studies by the group of Bahl [1] and Canfield [2] showed that the β -subunit contains a C-terminal sequence of 30 amino acids which does not occur in hLH and appears to represent a relatively extended unique structure exclusively occurring in the chorionic gonadotropin molecule. This feature may be of immediate practical interest provided that the C-terminal chain is sufficiently accessible within the structure of the entire hormone to allow interaction with antibodies raised against an adequate portion of the unique sequence.

We here describe the synthesis of an eicosapeptide related to the C-terminus of the β -subunit of HCG and

show that this peptide is capable of inducing antibodies which can interact with the entire HCG molecule.

2. Materials and methods

2.1. Peptide synthesis

The oligopeptides A, B and C (fig.1) were prepared in suitably protected form essentially by the two-phase method [3,4]. For subsequent condensation, peptides A and C were deprotected at the N-terminus, peptide B at the C-terminus. The deprotected peptides were purified by countercurrent distribution and exhibited acceptable elementary and/or amino acid analyses. Condensations of oligopeptides were performed by means of carbonyldiimidazole (CDI) in yields above 80% for each step. A scheme showing the combination of protecting groups, deprotection and condensation of oligopeptides to the protected eicosapeptide (EP) is shown in fig.2. The protected eicosapeptide (195 mg) was treated with HBr in glacial acetic acid in order

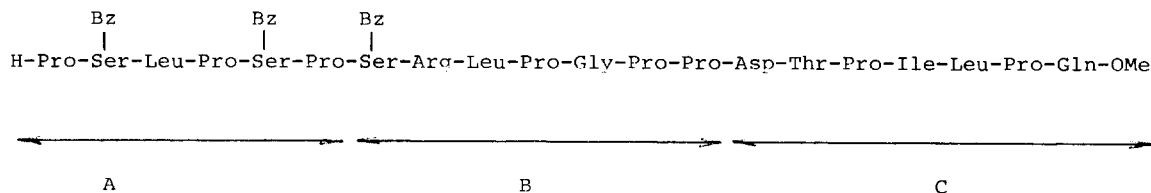


Fig.1. Synthetic peptides related to the C-terminus of β -HCG. The sequences A, B and C denote oligopeptides prepared by monotonous two-phase synthesis subsequently condensed to the eicosapeptide.

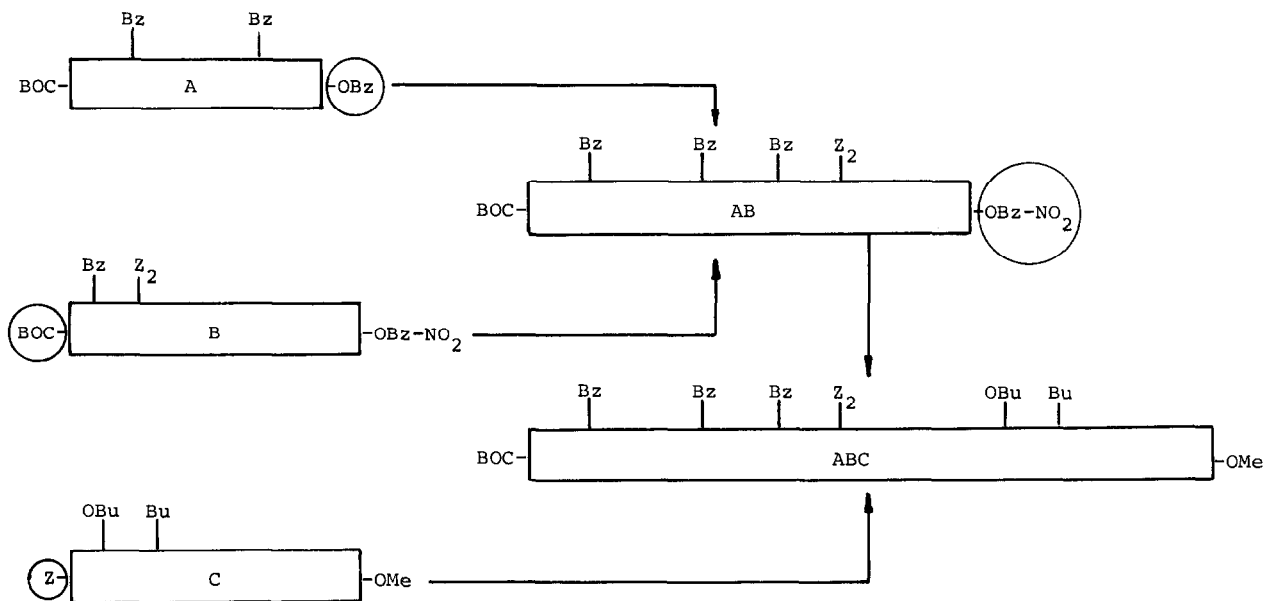


Fig.2. Synthesis of protected eicosapeptide by condensation of partially deprotected oligopeptides A, B, C. Removal of protective groups is indicated by circles.

to remove the protecting groups. The C-terminal methyl ester and the benzylethers on the serines are not split. The crude product was extracted with ether and passed through a Sephadex-G-25 column (1.4 X 40 cm) with H₂O as the eluant. The eluate was collected in 2.1 ml fractions. The material emerging first (fractions 12–17) was lyophilized to give 154 mg of fluffy white ninhydrin-positive powder. A second component eluted in fractions 23–38 was obtained in the form of a ninhydrin-negative yellow oil and not further analyzed.

2.2. Conjugation of eicosapeptide to protein (EP-HSA)

To 10 mg human serum albumin (HSA) in 1.0 ml 0.1 M sodium borate buffer pH 9.5, 0.02 ml toluylene-diisocyanate (TDIC) (Fluka AG., Buchs) in 0.18 ml CH₂Cl₂ was added and stirred at 0°C. After 30 min the mixture was centrifuged and the light phase was added to 10.75 mg deprotected, lyophilized EP. The solution was kept at 37°C for 4 hr and then passed through a Sephadex-G-25 column (1.4 X 40 cm) with H₂O as the eluant. The proteinaceous material eluted first was lyophilized (14 mg). Quantitative amino

acid analysis established that on the average 8.4 peptide chains were bound per protein molecule. In model experiments with less precious peptides it was established that the alcoholic hydroxyls do not significantly compete with the N-terminal amino group in the conjugation reaction.

2.3. Conjugation of HCG to dextran (HCG-DEX)

HCG (Sigma Ltd., St. Louis) was coupled to dextran (mol. wt 70 000) by means of cyanogen bromide according to standard procedures [5,6]. The conjugate was isolated by gel filtration.

2.4. Immunizations

Program A: Random-bred guinea pigs received 0.1 mg EP together with complete Freund's adjuvant (CFA) into each footpad. Intradermal boosts are given periodically. **Program B:** Rabbits (2 animals) received 1 mg EP-HSA with CFA into each footpad. Intravenous boosts are given periodically.

2.5. Immunological tests

Passive cutaneous anaphylaxis (PCA) was performed in guinea pigs according to standard procedures [7].

Skin reactions were quantitated by measuring skin thickness increase at the reaction site by means of a caliper [7].

3. Results

The synthesis of the eicosapeptide derivative related to the C-terminal sequence of the β -subunit of HCG (fig.1) was accomplished according to schedule by two-phase synthesis and CDI-condensation of oligopeptides A, B and C (fig.2). Probably due to the abundance of proline residues in the sequence poorly defined side-product formation was observed during preparation of oligopeptides and unusually frequent intermediary purification by countercurrent distribution became necessary. The final product obtained after gel filtration moved as a single zone on circular paper chromatograms (R_f 0.70 in 1-butanol–acetic acid– H_2O / 4:1:5; R_f 0.77 in 1-butanol–pyridine–acetic acid– H_2O / 15:10:3:12) and on paper electrophoresis (3 mm cathodic movement, trace at 11 mm anodic movement in 0.05 M phosphate buffer pH 7.4 at 9 V/cm for 130 min). Detection of zones was in all cases with ninhydrin and with J_2 -vapour. Analysis: Calcd. for $C_{116}H_{174}Br_2N_{24}O_{28}$ (2512.5): N, 13.37%. Found: (corrected for 12.6% ash): N, 13.39%. The amino acid composition of the peptide after hydrolysis with 5.6 N HCl at 115°C for 20 hr was: Asp: 1.1; Thr: 1.1; Ser₃: 2.6;

Table 1
Detection of antibodies by PCA three weeks after CFA-immunization of guinea pigs with C-terminal eicosapeptide of β -HCG

Serum tested from*	Reaction in mm ² blue area elicited by**	
	EP-HSA	HCG-DEX
animal No 1	480	330
animal No 2	0	10
animal No 3	130	80
animal No 4	540	150
animal No 5	0	20
non-immunized animal	0	10

* Sera (1:3) were injected intradermally into recipient guinea pigs. Elicitation was by intravenous administration of antigen conjugates.

** Average from 2 animals.

Table 2
Detection of antibody by PCA three weeks after CFA-immunization of rabbits with β -HCG-eicosapeptide conjugated to IISA

Serum and dilution*		Reaction in mm ² elicited by**	
		HCG-DEX	DEX
anti-EP-HSA	1:10	340	30
anti-EP-HSA	1:20	210	25
anti-EP-HSA	1:100	180	25
anti-HCG***	1:100	500	60
normal serum	1:5	35	25

* Sera were injected and tested as indicated in table 1.

** Average from 2 animals.

*** Commercial rabbit anti-HCG-antiserum produced for radioimmunoassay.

Pro₈: 8.4; Gln: 1.0; Ile: 1.1; Leu₃: 3.0; Arg: 0.7 and thus was in sufficient agreement with expected values. Evaluation of immunization of guinea pigs with EP (program A) and of rabbits with EP-IISA (program B) is shown in tables 1 and 2 respectively. It is apparent that 3 out of 5 guinea pigs showed positive PCA reactions 3 weeks after primary immunization. The reactions could be elicited with either EP-HSA or with HCG-DEX. The rabbit antiserum was also positive in PCA elicited with HCG-DEX but expectedly weaker than a commercial (late) antibody against HCG. Six weeks after priming the guinea pigs were skin tested with EP (10,20,50 and 100 μ g intradermally). The 5 animals showed positive antibody-mediated immediate reactions between 0.5 and 1.3 mm skin thickness increase with the higher doses.

4. Discussion

The eicosapeptide synthesized according to the sequence shown in fig.1 contains 3 benzylethers on serines and a methylester on the C-terminal glutamine. The benzylethers are considered as substitutes for the short oligosaccharide chains bound to the serines within the natural sequence according to Bahl [1]. The eicosapeptide in unbound form as well as after conjugation to protein was clearly found to be immunogenic. This implies that sufficiently defined and rigid structures are present in the synthesized sequence to serve

as antigenic determinants. Antibodies raised in guinea pigs or rabbits were shown by PCA to react with the entire HCG molecule. In the PCA tests conjugated antigens (EP-HSA and HCG-DEX) instead of free antigens were used in order to circumvent eventual difficulties in elicitation. It is well established that immunochemical multivalency and suitable steric arrangements of antigenic determinants are prerequisites for successful PCA elicitation in cases where, like in the present one, only a small number of distinct determinants are involved. However, recent preliminary results show that our PCA reactions can also be elicited with unconjugated HCG. These immunological results show that the C-terminal chain of the β -subunit is sufficiently accessible within the structure of the entire glycoprotein hormone to allow interaction with antibodies directed against determinants of the unique C-terminal chain of HCG.

Applications of this result can be envisaged in at least two different fields. C-terminus peptides may be used for devising highly specific vaccines capable of immunizing the female organism against placental HCG and thus in fact against pregnancy. The feasibility of such an approach has been made likely by Stevens [8,9]. Furthermore, C-terminus-specific antibodies raised in suitable species could be used in radioimmune binding tests for the highly specific quantitation of HCG and thus serve as satisfactory tools for diagnosing early pregnancy as well as a variety of forms of malignancy [10,11].

The present synthetic sequence is probably near the minimal chain length adequate for application in the potential fields of interest. We have prepared a C-terminal tetracosapeptide which according to preliminary study does not differ significantly from the eicosapep-

tide in immunological tests. On the other hand C-terminal chains of more than 30 aminoacids prepared by the group of H. D. Niall seem to contain at least one additional antigenic determinant [9].

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