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Antibodies to the Carboxyl-Terminal Fragment of Human Chorionic Gonadotropin β-Subunit: Characterization of Antibody Recognition Sites Using Synthetic Peptide Analogues†

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ABSTRACT: The immunochemical specificities of an antiserum (H-93) generated by immunization of a rabbit with a bovine serum albumin conjugate of a unique COOH-terminal tricosaglycopeptide fragment (residues 123–145) isolated after tryptic digestion of the S-carboxymethylated, desialylated β-subunit of human chorionic gonadotropin (hCG) were analyzed systematically with a radioimmunoassay system. Using [^{125}I]hCG as labeled antigen, a series of 32 synthetic peptides of various lengths, analogous to the polypeptide sequence of the native antigen, was evaluated for antigenic recognition by the antiserum. Immunological cross-reactivity with the dipeptide, Pro-Gln, but not with Gln or Pro-Glu was observed. The degree of immunoreactivity increased with increasing chain length and reached a plateau at the pentadecapeptide, which was equipotent with a highly purified hCG. The dose–response curves of hCG and the pentadecapeptide or longer synthetic peptides corresponding to the COOH-terminal peptide were superimposable. Thus, the antibody recognition was shown to reside at the last 15 amino acid residues of the COOH-terminal peptide of hCGβ. Evidence is also presented to indicate that a free COOH terminus is not essential for immunological cross-reactivity. Although cross-reactivity increased most significantly over the peptide sequence Arg-Leu-Pro-Gly (residues 133–136), two peptides containing this sequence, but lacking the last portion of COOH-terminal peptide, showed significantly lower (residues 125–137) or insignificant (residues 131–137) cross-reactivity. These results demonstrate that the Pro-Gln dipeptide segment is one of the important recognition units for the antibody and that the addition of the other 13 residues enhances the cross-reactivity by 5×10^4. Four more antisera produced in rabbits treated with the identical immunogen exhibited binding characteristics very similar to that of the H-93 antiserum. These studies describe the nature of the sites of antibody recognition and provide the basis for the high degree of specificity for hCG of the H-93 antiserum without cross-reactivity to structually similar hLH.

Human chorionic gonadotropin (hCG)[1] is a glycoprotein hormone of pregnancy, normally synthesized and secreted by the placenta and detectable in peripheral serum and urine coincident with implantation of the blastocyst 7–9 days after fertilization (Braunstein et al., 1973; Kosasa et al., 1973; Landsman and Saxena, 1976). Physiologically hCG stimulates enhanced and prolonged secretion of progesterone by the corpus luteum into early pregnancy and may stimulate development of fetal gonads during mid-gestation (Clements et al., 1976). HCG is measurable in serum and urine throughout pregnancy and, because of its long circulatory half-life, has been detected more than a week into the postpartum interval (Faiman et al., 1968; Jaffe et al., 1969). Except for the presence of hCG in the fluids and tissues of persons bearing neoplasms, trophoblastic or otherwise (Goldstein et al., 1974; Vaitukaitis et al., 1976), this glycoprotein had been regarded as a hormone exclusive to pregnancy. However, recent findings indicate the presence of an hCG-like substance in testicular tissue of normal men (Braunstein et al., 1975), in urinary and pituitary extracts of normal subjects (Chen et al., 1976b), as well as in media of bacterial populations (Livingston and Livingston, 1974; Cohen and Strampp, 1976). Accordingly, it is important to extend these new observations employing a specific hCG assay system that can discriminate hCG from hLH.

HCG consists of dissimilar α and β subunits (Canfield et al., 1971), much like other glycoprotein hormones, including hLH, hFSH, and hTSH. Although the α subunits of all these hormones have very similar primary structures, their respective β subunits are structurally different, thereby imparting expressions of specific hormonal activities (Pierce, 1971). However, the β subunits of hCG and hLH show remarkable similarity in amino acid sequence (Morgan et al., 1975). Even the nature of their biological activities is nearly analogous, in that they compete for the same receptors in both testicular (Catt et al., 1972) and ovarian tissues (Lee and Ryan, 1972;

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[1] Abbreviations used are: hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; hTSH, human thyroid stimulating hormone; hFSH, human follicle stimulating hormone; RIA, radioimmunoassay; Boc-, *tert*-butyloxycarbonyl-; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; BSA, bovine serum albumin.

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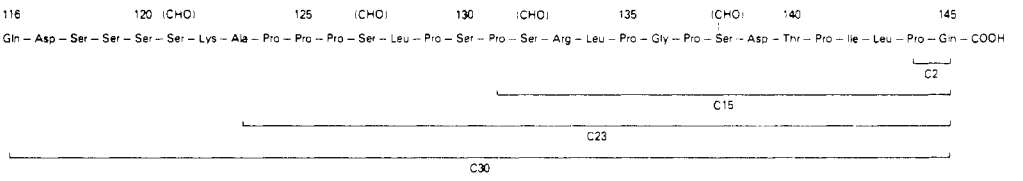

FIGURE 1: Amino acid sequence of the unique COOH-terminal portion of hCGβ subunit (Morgan et al., 1975). CHO represents carbohydrate moiety. In the synthetic peptides, carbohydrate is not present. NH_2 terminus of C30 was acetylated.

Haour and Saxena, 1974) and stimulate steroidogenesis alike (Dufau et al., 1971). Nevertheless, hCGβ possesses an extended, unique COOH-terminal peptide of approximately 30 amino acid residues not present on hLHβ. Taking advantage of this unique structural feature of hCGβ, hCG specific antisera were generated by immunizing rabbits with the COOH-terminal tricosaglycopeptide conjugated to BSA. This fragment was isolated after tryptic digestion of an S-carboxymethylated, desialylated preparation of purified hCGβ (Louvet et al., 1974). These antisera, most notably H-93 with the highest titer, distinguished fully hCG from hLH (Chen et al., 1976a,b). In preliminary investigations using antisera to this COOH-terminal peptide and synthetic peptide analogues to the COOH-terminal sequence of hCGβ, we found that the antibody recognition sites on hCG resided exclusively among the last 15 COOH-terminal amino acid residues of hCGβ (Chen et al., 1976b).

In this report, we describe detailed characterization of the antibody recognition sites employing synthetic peptide analogues between the dipeptide and triacontapeptide analogous to the native antigen. Five antisera raised against the COOH-terminal desialylated glycopeptide (residues 123–145) of hCGβ were evaluated. The findings presented characterize the primary site of antigenic recognition, as well as the enhancement in affinity which accompanied progressive increases in the peptide chain elongated to the pentadecapeptide.

Materials and Methods

Boc-L-amino acid derivatives were either purchased from Beckman Inc., Palo Alto, Calif., or prepared according to the procedure of Schnabel (1967). Other chemicals were obtained from the various commercial sources in parentheses: chloromethylated polystyrene–divinylbenzene resin, Bio-Beads SX1, 1.25 mequiv of chlorine/g, 200–400 mesh (Bio-Rad Labs, Richmond, Calif.); Sephadex G-50 and DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc.); TFA, DIEA, and *N,N'*-dicyclohexylcarbodiimide (Pierce, Rockford, Ill.). All solvents were analytical grade.

A highly purified hCG preparation (CR119) containing 11 600 IU/mg (Second International Standard) was used as standard hCG preparation (Morgan et al., 1974). Three synthetic peptides (hCGβ residues 125–130, hCGβ 131–137, and hCGβ 125–137) were generously supplied by Dr. V. C. Stevens, Ohio State University, Columbus, Ohio. The amino acid analyses were carried out either on a Beckman-Spinco Model 121 M or JEOL Model JLC-6AH amino acid analyzers after hydrolysis of the samples in 6 N HCl for 24 h in evacuated sealed tubes at 110 °C. The following solvent systems were used for thin-layer chromatography: *n*-BuOH:AcOH:H_2O (4:1:1); *n*-BuOH:pyridine:AcOH:H_2O (4:1:1:2); 2-propanol:1 N AcOH (2:1).

Synthesis of the HCGβ-COOH-Terminal Peptides. The synthesis of the protected polypeptide resins corresponding to the COOH-terminal polypeptide sequence (C30,[2] residues 116–145) of hCG β subunit (Figure 1; Morgan et al., 1975), and 28 intermediate peptides, were carried out manually by the modified stepwise solid phase procedure of Merrifield (1964). Boc-L-Gln was esterified to the chloromethylated 1% cross-linked polystyrene resin by the cesium salt method (Gisin, 1973). The synthesis was started with 25 g of Boc-Gln–resin (0.44 mequiv of Gln/g of resin). The α-amino group of all amino acids was protected by the Boc group. The side chain functional groups of Asp, Glu, Ser, and Thr were protected by the benzyl group, the ϵ-amino group of Lys was blocked by the carbobenzyloxycarbonyl group and the guanidino side chain of Arg was blocked by the tosyl group. Boc group was removed by treatment with 50% of TFA in CH_2Cl_2 and neutralization was carried out with 10% DIEA in CH_2Cl_2. The "preformed symmetric anhydride" method (Hagenmaier and Frank, 1972) was used in all couplings except Boc-Gln, which was coupled as its *p*-nitrophenyl ester. After Gln-29 cycle of synthesis, Boc group was removed and the peptide on resin was acetylated with acetic anhydride (Stewart and Young, 1969). For cleavage of intermediate peptides from the resins, aliquots of the protected peptide resin, after each cycle of the synthesis, were taken out and treated with HF (Sakakibara and Shimonishi, 1965) at 0 °C in the presence of anisole. After removal of HF and washing of the residue with ethyl acetate, the dried residue was extracted with 0.2 N acetic acid under gentle stirring at room temperature for 3 h. The filtrate recovered from the resin was lyophilized.

Purification of the Peptide. Most of the peptides were used for RIA directly after HF treatment and lyophilization without purification. C12 and C28 were further purified as follows: crude peptide from HF cleavage was subjected to gel filtration on a Sephadex G-50 column (1.7 × 200 cm) in 0.2 N acetic acid. Pooled fractions from the major peak were evaporated under reduced pressure to a small volume and were subjected to DEAE-Sephadex A-25 chromatography (column, 1.7 × 80 cm) with linear gradient elution using 0.01 M to 2.0 M ammonium bicarbonate buffer (pH 8.0). The material from the major peak was collected and lyophilized.

Production of Antisera. All antisera used were generated in rabbits immunized with COOH-terminal tricosaglycopeptide from the S-carboxymethylated, desialylated hCGβ conjugated to BSA. H series of antisera H-93, H-94, H-95, and H-100 (Chen et al., 1976b) and JPL-8 from the JPL series (Louvet et al., 1974) produced and reported previously were used in these studies.

Radioimmunoassay. The reaction mixture contained the following reagents which were added to 1.0 × 7.5 cm glass test tubes in order: (1) 5 to 200 μL of sample solution to be assayed,

[2] For simplicity, peptides elongated from the COOH-terminal amino acid Gln according to the amino acid sequence of hCGβ proposed by Morgan et al. (1975) are expressed in number with prefix C. Example C2 is dipeptide Pro-Gln; C3, Leu-Pro-Gln, - - -, etc.

TABLE I: Amino Acid Composition[a] of Synthetic Carboxyl-Terminal Peptides of HCGβ Subunit.

Amino acid	Peptide														
	C2	C4	C6	C8	C10	C12	C14	C16	C18	C20	C22	C24	C26	C28[c]	C30
Asp				0.98	0.97	0.98	0.98	0.97	0.99	0.97	0.96	0.97	0.98	0.98	1.96
				(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(2)
Thr			1.08	0.97	0.98	0.98	0.97	0.96	0.97	0.96	0.96	0.96	0.96	0.98	0.97
			(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
Ser				0.97	0.92	0.87	1.90	2.79	2.80	3.52	3.60	3.52	5.57	7.92	7.79
				(1)	(1)	(1)	(2)	(3)	(3)	(4)	(4)	(4)	(6)	(8)	(8)
Glu	1.20	1.22	1.18	1.17	1.15	1.09	1.10	1.13	1.08	1.18	1.17	1.18	1.14	1.02	2.08
	(1)[b]	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(2)
Pro	1.12	1.12	2.06	2.01	3.00	3.97	4.18	5.18	6.14	7.18	8.92	8.89	8.92	9.05	8.89
	(1)	(1)	(2)	(2)	(3)	(4)	(4)	(5)	(6)	(7)	(9)	(9)	(9)	(9)	(9)
Gly					1.02	1.08	1.05	1.07	1.00	0.98	0.97	1.07	1.07	1.04	1.10
					(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
Ala												0.98	0.98	0.99	1.06
												(1)	(1)	(1)	(1)
Ile		1.00	0.98	0.98	0.99	0.98	0.97	0.98	0.98	0.97	0.98	0.97	0.97	1.04	1.02
		(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
Leu		1.13	0.97	0.99	0.98	1.89	1.88	2.07	2.92	2.98	2.96	3.05	2.98	3.03	3.08
		(1)	(1)	(1)	(1)	(2)	(2)	(2)	(3)	(3)	(3)	(3)	(3)	(3)	(3)
Lys												1.18	1.10	1.08	1.12
												(1)	(1)	(1)	(1)
Arg							1.12	1.08	1.10	1.15	1.10	1.10	1.08	1.03	1.18
							(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
Total	(2)	(4)	(6)	(8)	(10)	(12)	(14)	(16)	(18)	(20)	(22)	(24)	(26)	(28)	(30)

[a] Values expressed as residues per peptide. [b] Numbers in parentheses indicate expected integral values. [c] Data obtained from purified C28 (Figure 2).

or of hCG–CR119 solution in 0.01 M phosphate, 0.15 M NaCl, 0.1% BSA, pH 7.8; (2) the buffer (0.01 M phosphate, 0.15 M NaCl, 2.5% normal rabbit serum, pH 7.8) to make a total final volume of 1 mL; (3) 100 μL of 0.1 M EDTA, 0.1% NaN_3, pH 7.8; (4) 200 μL of [^{125}I]hCG–CR119 (85–90 μCi/μg, approximately 25 000 dpm/tube in the buffer); (5) 200 μL of the antiserum diluted to 1:1600 also in the buffer. Tubes were incubated initially at 37 °C for 2 h, then at 4 °C for an additional 12–16 h. Second antibody (sheep anti-rabbit γ globulin serum) was added at the end of that time, and incubation was continued for another 4–6 h at 4 °C. Bound and free antigens were separated by centrifugation and aspiration of the supernatant. All assays were carried out using either 6 doses in triplicate (standard, hCG-CR119) or 5 doses in duplicate (unknown samples). Computation of potencies and calculations and comparisons of slopes of dose–response curves were done by a modified computer program described by Rodbard (1974).

Results

Synthesis and Characterization of Peptides. All synthetic peptides gave the expected molar ratio on amino acid analysis as shown in Table I. Although individual peptides except C12 and C28 were not purified due to insufficient aliquots of the protected peptide resin available, the good agreement in amino acid compositions of all peptides and the gel chromatographic pattern of C28 prior to purification (Figure 2A) indicate the near correctness of coupling at each step during synthesis. The advantage of "preformed symmetric anhydride" method according to Hagenmaier and Frank (1972) and the absence of Met, Cys, His, Tyr, and Trp may contribute to this effect. Thus, the impurities shown in Figure 2B most probably would arise from incomplete deprotection of side chain functional groups.

An intermediate peptide, C12, showed homogeneous elution patterns in either Sephadex G-50 gel chromatography or

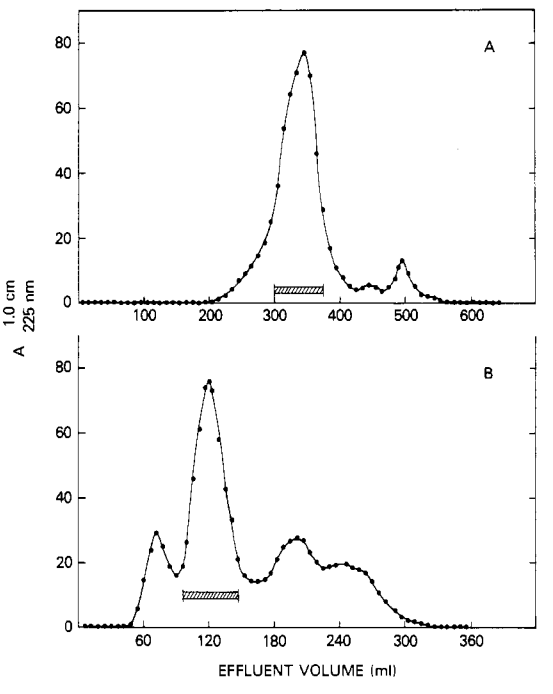

FIGURE 2: Elution profiles of a synthetic peptide, C28. (A) Peptide (360 mg) was dissolved in 2 mL of 0.2 N acetic acid and applied to a column of Sephadex G-50 (1.7 × 200 cm). Elution was performed with 0.2 N acetic acid. (B) Pooled fractions from shaded area of A were applied to a column of DEAE-Sephadex (1.7 × 80 cm). Elution was performed with linear gradient using 0.01 M to 2.0 M ammonium bicarbonate, pH 8.0.

DEAE-Sephadex A-25 ion-exchange chromatography. Under the same conditions, C28 was purified in two steps as shown in Figure 2. The purity of this peptide was assessed showing only one major spot by thin-layer chromatography in three systems except on very heavy loading of the sample and by the amino acid composition. The peptide C12 was subjected to

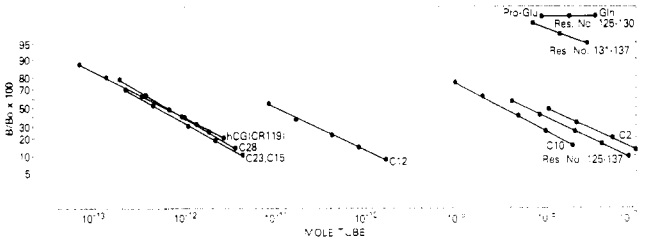

FIGURE 3: Dose-response curves of synthetic peptides analogous to the COOH-terminal sequence of hCGβ in a radioimmunoassay system based on an antiserum to hCGβ-COOH-terminal peptide (H-93) diluted 1:8000. B = cpm bound in the presence of [^{125}I]hCG and unlabeled ligand; B_0 = cpm bound in the presence of [^{125}I]hCG alone.

Edman degradation (Edman, 1956) and found to be consistent with the expected sequence for the COOH-terminal peptide of hCGβ (Morgan et al., 1975).

Interaction and Specificity of Anti-HCGβ-COOH-Terminal Peptide Serum for Synthetic Peptides. The interaction of [^{125}I]hCG with the anti-hCGβ-COOH-terminal peptide serum and its inhibition by a series of the synthetic peptide analogues to the unique COOH-terminal peptide of hCGβ were studied using RIA. The antiserum (H-93) was appropriately diluted with 0.01 M phosphate + 0.15 M NaCl, pH 7.8, containing 2.5% normal rabbit serum, so that between 20 to 30% of the added [^{125}I]hCG in the absence of any nonlabeled ligand were bound to the antibody. The nonspecific binding was determined by the amount of [^{125}I]hCG precipitated in the absence of the antiserum and was always below 2.5% of the total amount of radioactivity. All data have been corrected for this nonspecific binding. The dose-response curves of representative synthetic peptides and the highly purified hCG (CR119) for the inhibition of binding of [^{125}I]hCG to antiserum (H-93) are shown in Figure 3. The parallelism between lines of the synthetic COOH-terminal peptides and that of highly purified hCG was remarkable, especially with lengthening peptide chains, indicating that C15 and longer peptides competed equally with native hCG for binding by the antiserum. Also, Figure 3 shows that the dipeptide, C2, Pro-Gln, inhibited [^{125}I]hCG binding, whereas neither Pro-Glu, nor residues 125–130 showed cross-reactivity. A longer intermediate segment, residue 125–137, yielded cross-reactivity 2×10^{-4} times that of C21 (residues 125–145) which contains this segment and sequence as its NH_2-terminal portion. Likewise, a shorter intermediate segment, residues 131–137, was found by extrapolation to be 2×10^{-6} times that of C15 (residues 131–145) which is hardly significant, since all three points gave B/B_0 ratio larger than 0.95 (Figure 3).

The demonstration of binding of Pro-Gln by the H-93 antibody, but not Pro-Glu or Gln, alone indicates Pro and the γ-$CONH_2$ on the Glu residue might be essential to the initiation of antigen–antibody recognition. As to whether the free α-COOH group is necessary, we have demonstrated previously (Chen et al., 1976b) that peptidation of the α-COOH group with *S*-methylcysteinylamide did not interfere with immunological cross-reactivity. In addition, examination of a polymerized COOH-terminal peptide,[3] synthesized according to the sequence proposed by Carlson et al. (1973), which contained three additional residues (-Ser-Leu-Pro) following Pro-Gln, showed comparable cross-reactivity. Therefore, it appears that the Pro-Gln unit constitutes the primary locus of antigenic recognition by antiserum to the COOH-terminal peptide of hCGβ, even when the α-COOH group is not free.

[3] Gift from Drs. S. Koide and Y. Y. Tsong, The Population Council, New York, N.Y.

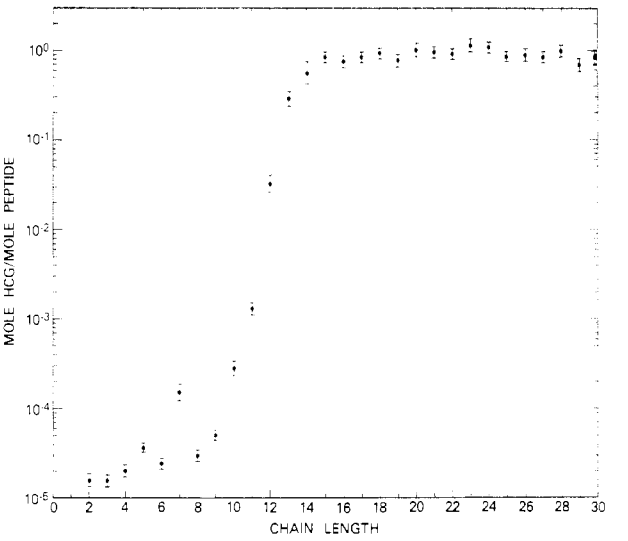

FIGURE 4: Competitive binding activities of synthetic peptides analogous to the COOH-terminal sequence of hCGβ in a radioimmunoassay system with [^{125}I]hCG and an antiserum to the hCGβ COOH-terminal peptide (H-93). The ordinate indicates moles of unlabeled hCG required to inhibit equally [^{125}I]hCG binding to antiserum per mole of peptides. Values of 95% confidence limit for each point are indicated by two horizontal bars.

Figure 4 exhibits the pattern of competitive binding by a series of synthetic peptides (C2–C30), relative to hCG (CR119), in the H-93 RIA system. As mentioned previously, the peptide unit displaying detectable inhibition of [^{125}I]hCG binding under these RIA conditions was C2, Pro-Gln. C3 to C8 did not enhance appreciably the binding of these synthetic peptides to the H-93 antiserum. Incorporation of Pro to C8 led to approximately a twofold increase in binding. At C10, the inhibition of binding had increased an additional fivefold and continued to rise markedly with each additional residue in peptide chain, until a plateau was reached after incorporating additional residues up to C15. At the plateau, it can be seen that C15 is about 5×10^4 times as potent as C2. The absence of antigenic recognition among residues distal to the C-terminal region (C16 and longer synthetic peptides) is intriguing. Perhaps, since BSA is an acidic protein and was used as a carrier agent, the NH_2 group of the peptide and the COOH group of BSA are the most probable points of conjugation. Accordingly, it may be that the NH_2-terminal region of the native antigen fragment was sterically hindered by its attachment to BSA, thereby preventing these amino acid residues (C16–C23) from participating as an antigenic region. However, intrinsic inertness in eliciting antigenicity from this segment of peptide, not involving the carrier protein, cannot be ruled out. Clearly, C15, as well as the longer synthetic peptides, was equipotent to hCG (CR119) when binding to hCGβ-COOH-terminal peptide sera. This finding indicates that the peptide moiety, rather than carbohydrate components, dictates the sites of antigenic recognition.

The dramatically increased binding produced by elongating C9 at the NH_2 terminus with Gly, Pro, Leu, and Arg, in sequence, is noteworthy. The addition of Leu to C11 was accompanied by the most striking enhancement in immunoreactivity, suggesting that one of the sites critical to interaction between native hCG and the H-93 antiserum may involve hydrophobic bonding at this locus. However, the positive charge on the guanidino group is not essential, since the same enhancement of immunoreactivity was observed when the guanidino group of Arg was nitrated (Chen et al., 1976b).

Upon comparing to hCG the established amino acid se-

quences of other glycoprotein hormones, it is apparent that the sequence, Arg-Leu-Pro-Gly, exists also in the middle of sequence other than the COOH-terminal region of both hCGβ (residues 68–71, Morgan et al., 1975) and hLHβ (residues 69–70, Sairam and Li, 1975; residues 66–69, Shome and Parlow, 1973). Nevertheless, the heptapeptide (residues 131–137) which contained this tetrapeptide segment (Arg-Leu-Pro-Gly) was not significantly immunoreactive by itself in the H-93 RIA system (Figure 3). Uniformly, we have found that highly purified hLH preparations do not react in this RIA, despite such similarities in the structure of the β subunits of hCG and hLH. Perhaps because this common internal tetrapeptide exists adjacent to one of the disulfide bridges the resultant conformation may further negate otherwise potential immunoreactivity. Therefore, we suggest that antigenic recognition of hCG by the H-93 antiserum may reside exclusively at those sequences which make up the COOH-terminal pentadecapeptide and in this way imparts the observed high degree of specificity for hCG and showed no detectable cross-reactivity with hLH.

Uniformity of Antigenic Recognition among Anti-HCGβ-COOH-Terminal Peptide Sera. Among ten rabbits immunized with the COOH-terminal glycopeptide of hCGβ conjugated to BSA, four produced appreciable titers for the binding of [^{125}I]hCG in RIA. The final dilutions to achieve 20 to 30% binding of the total [^{125}I]hCG for these four sera (H-93, H-94, H-95, and H-100) were 10^4 through 10^3. Also, antiserum JPL-8, made similarly the previous year (Louvet et al., 1974), was included for comparative studies. All antisera were examined for their binding specificities in the same manner as described above for the H-93 antiserum. The results are summarized in Figure 5. Although data for each antiserum was obtained from one assay, experiments with different antisera were carried out separately. Clearly, all five antisera displayed very similar binding characteristics and specificity toward the series of synthetic peptides.

It is established that when an immunogen is injected into several recipients it may elicit antisera having very different specificities of antigenic recognition (Benjamini et al., 1968). In the present study we found that four of ten rabbits made rather uniform antisera, each displaying virtually identical specificities of antigenic recognition. That is, they are all hCG specific and manifest immunoreactivity with two loci on the COOH-terminal peptide of hCGβ, namely, hydrogen bonding at the terminal dipeptide and hydrophobic bonding at the middle position as discussed previously. The same immunological specificity was also displayed by antiserum, JPL-8, obtained by an independent series of immunization (Louvet et al., 1974). More recently, we have produced antiserum H-114 in a rabbit immunized with C30 conjugated to bovine thyroglobulin.[4] This antiserum not only showed similar sensitivity toward hCG, but also revealed the same specificity for antigenic sites residing in the pentadecapeptide region of hCGβ, identical with that of H-93 antiserum.

Discussion

The capacities of various immunological methods to detect differences in antigenicity between structurally related antigens vary considerably. The RIA method employed here is one of great precision compared with microcomplement fixation or quantitative precipitation. The superiority of RIA depends on the low concentration of labeled antigen and subsequently that of antibody required. Thus, only the most avid portion of the total antibody population is permitted to react, and a small difference in binding affinity between two antigens may be amplified into a relatively large difference in immunoreactivity which in turn provides a highly sensitive and reliable measurement. However, precautions must be undertaken in validation of RIA measurements and interpretation of results. Comprehensive treatments in these regards, based on theoretical and experimental aspects derived from statistical and kinetic analyses, have been examined extensively by Rodbard (1974).

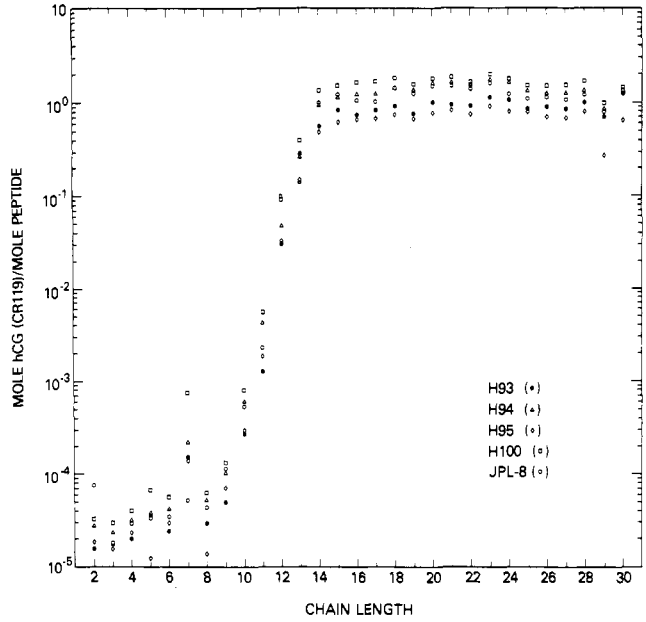

FIGURE 5: Competitive binding activities against [^{125}I]hCG of synthetic peptides in a radioimmunoassay system with various antisera to hCGβ COOH-terminal peptide (H-93, H-94, H-95, H-100, and JPL-8). The ordinate and abscissa are similar to those of Figure 4.

The purity of synthetic peptides used in the present studies deserves close attention. As was discussed in the Results section, impurities would result most likely from incomplete deprotection of side chain functional groups rather than incomplete coupling or branching during synthesis. However, these kinds of impurities in the peptide preparations did not appear to have an overall effect on binding since purified C28 showed cross-reactivity with hCG indistinguishable from that of the unpurified peptides (C15 to C30). Furthermore, equal potency of binding to the antibodies between hCG and C15 or longer peptides on a mole per mole basis is highly unlikely to be coincident. Nevertheless, studies with purified C14 and C15 are desirable, and a new series of synthesis will be required to achieve this goal.

Determination of interaction between anti-hCG-β-COOH-terminal peptide sera with a series of synthetic peptide analogues clearly indicates that the antibody recognition sites of the COOH-terminal region reside within the pentadecapeptide segment. The importance of the COOH-terminal dipeptide, Pro-Gln, in initiating antigenic recognition is evident. The peptide analogues lacking COOH-terminal portion, including Pro-Gln, showed four to six orders of magnitude lower cross-reactivity. Apparently, this is the basis of the ability of the H-93 antiserum to distinguish hCG from other glycoprotein hormones. Perhaps a similar COOH-terminal amino acid sequence exists in the chorionic gonadotropins of chimpanzee and gorilla, but not that of orangutans, baboons, macaques, and marmosets, since the former ones are antigenically indistinguishable from hCG (Chen and Hodgen, 1976).

[4] Matsuura, S., Chen, H. C., and Hodgen, G. D., unpublished.

The reproducible antigenicity of the tricosaglycopeptide immunogen employed in making H-93 antiserum may relate to the shortness of the peptide and its limitation to two antigenic regions. However, all these antibodies to COOH-terminal portion of hCGβ are one order of magnitude lower in affinity than typical antibodies elicited either from hCGβ or intact hCG (Vaitukaitis et al., 1972; Chen et al., 1976b). In much the same way, antibodies to an NH_2-terminal fragment of sickle β-globin were of lower affinity than those to the whole sickle β-globin (Curd et al., 1976).

It is known that the β subunits of hCG and hLH are very similar and unlike those of other glycoprotein hormones (Pierce, 1971). Accordingly, hCG and hLH may be indistinguishable by gonadal receptors (Catt et al., 1972). Since their greatest structural difference is the extended sequence of 30 amino acid residues on the COOH-terminal end of hCGβ, perhaps this unique peptide does not participate in receptor binding and subsequent steps initiating steroidogenesis. Indeed, in vivo biological neutralization experiments using anti-hCGβ-COOH-terminal peptide serum did not neutralize hCG activity despite binding to hCG (Louvet et al., 1974; Chen and Hodgen, 1976). Conversely, antisera to hCG or hCGβ, or hCGα, did neutralize the biological activity of hCG (Hodgen et al., 1973). It appears that the unique COOH-terminal peptide of hCGβ, or at least its last 15 residues, may extend outward as an appendage.

In conclusion, we have undertaken a systematic approach in characterizing antisera to the unique COOH-terminal region of hCGβ using a series of synthetic peptides analogues. Examination of the enhancement in antigen-antibody affinity with each added amino acid demonstrates the nature of antibody recognition, providing the basis of high specificity for hCG by antisera to the COOH-terminal peptide of hCGβ, without cross-reactivity to hLH.

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