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THE 20,000-DALTON VARIANT OF HUMAN GROWTH HORMONE: LOCATION OF THE AMINO ACID DELETIONS

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SUMMARY: The 20,000-dalton variant of human growth hormone lacks a sequence of 15 amino acids normally found in the hormone. The deletion involves residues 32 through 46. The remainder of the molecule is identical to the larger form of the hormone. These results suggest that the variant is the product of a deletion mutation in the growth hormone gene.

During a study of the heterogeneity of human growth hormone (hGH, M_r = 22,000) a 20,000-dalton form was detected (1). Subsequently, the newly recognized lower molecular weight variant (20K) was isolated and partially characterized (2). The substance promotes growth in rats to about the same extent as does hGH but it is less reactive toward antibodies to hGH. Structure studies indicated that the variant differs from hGH by having 15 to 20 fewer amino acids in the region of residues 20 through 64. Additional work has now permitted us to identify the exact location of the deletion.

MATERIALS AND METHODS

Hormone preparations. The procedures for the isolation of 20K (2) and hGH (3) have been reported.

Analytical electrophoresis. Electrophoresis was done (4) in a 6.5% polyacrylamide gel containing 6M urea and 0.1% sodium dodecyl sulfate (SDS). Samples from the Sephadex/10% acetic acid columns, usually 50-100 µl, were dried in a vacuum desiccator and then dissolved in 75 µl of the SDS/urea sample solution (4) and applied to the electrophoresis gels.

Preparation of the core peptides. Our approach to determination of the location of the deletion in 20K was to isolate the large, two-chain disulfide peptide corresponding to sequence 20 through 64 and 135 through 167 in hGH. This would provide the region where we believed 20K differed from hGH. The large peptide is referred to as the core peptide (see Fig. 1).

a) *Citraconylation and digestion with trypsin.* A 25 mg sample of hormone was dissolved in 5 ml of 0.05M NH_4HCO_3 (pH 8.3) and treated with 50 µl of citra-

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conic anhydride (5). Without further treatment, 0.5 mg of trypsin-TPCK was added and the mixture was incubated at 37° for 18 hours. The sample was then lyophilized.

b) *Isolation of the core peptide.* The peptides in the trypsin digest of the citraconylated hormone were separated at room temperature on a column (1.5 x 160 cm) of Sephadex G-50 Superfine equilibrated with 10% acetic acid. The digest was applied in 1.5 ml of 10% acetic acid; 3 ml/tube was collected.

Fig. 2*a* shows an elution pattern obtained with a trypsin digest of citraconylated 20K. A similar pattern was obtained with hGH except that the core peptide eluted with a maximum peak at tube 62. SDS/urea electrophoresis was used to locate the core peptides. Yields were 8 mg for the hGH peptide and 5.6 mg for the 20K peptide. Chromatography in acetic acid removed the citraconyl blocking groups.

Oxidation and trypsin cleavage of the core peptides. The core peptide was oxidized with performic acid (6) and lyophilized. The preparation was next dissolved in 0.5 ml of 0.05M NH_4HCO_3, pH 8.3. Trypsin-TPCK was added at an enzyme: peptide ratio of 1:100 (w/w), the mixture incubated at 37° for 5 hours and then lyophilized. The resulting peptides were separated on a column (0.9 x 60 cm) of Sephadex G-25 Superfine developed with 10% acetic acid; 1.5 ml fractions were collected. The elution pattern obtained with the 20K core is shown in Fig. 2*b*. Amino acid analysis and 3 cycles of microsequencing indicated that the first peak (T_1-T_2) contained the desired peptide.

Digestion with chymotrypsin. The T_1-T_2 peak (Fig. 2*b*) was treated with chymotrypsin in 0.05M NH_4HCO_3 for 4 h at 37° with an enzyme:peptide ratio of 1:100 (w/w). The digest was applied directly to thin layer plates of cellulose for peptide mapping.

Thin layer peptide mapping. The procedure has been described in detail (2). About 100 µg of sample was applied to cellulose coated (0.1 mm) plates. The peptides were located by spraying with 0.001% fluorescamine in acetone. The peptides were scraped from the plate and eluted with either 50% pyridine if the peptide was to be sequenced or 6N HCl when total amino acid analysis was to be performed.

Sequence analysis. The micro manual method of Chang *et al.* (7) which permitted analysis of nmoles of peptide was used.

RESULTS

Size characteristics of the core peptides. The diagramatic representation of the hGH molecule (8) shown in Fig. 1 shows the location of the arginine and lysine residues. By preventing trypsin cleavage at the citraconylated lysines (Lys-X), a large tryptic peptide was produced. In hGH this was the two-chain peptide made up of residues 20 through 64 connected to 135 through 167 by disulfide linkage. A peptide such as this, denoted as the core peptide, was isolated from both hGH and 20K by Sephadex chromatography. The elution pattern for 20K is shown in Fig. 2*a*. SDS/urea electrophoresis indicated that the 20K core peptide had a M_r = 7,000; the hGH peptide, 9,000. Under reducing conditions the 7K peptide dissociated to a broad band with a molecular weight near 3,500. The 9K

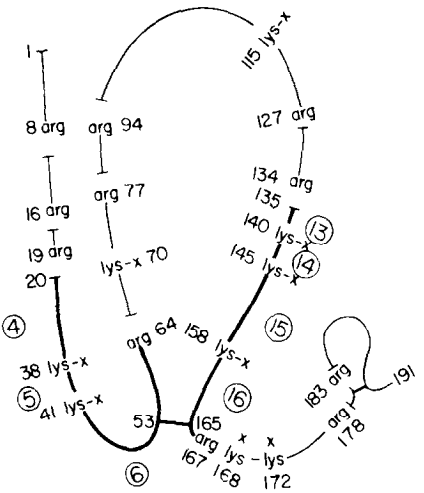

Fig. 1. *Diagramatic representation of the hGH molecule.* The tryptic peptides of the core peptide (20 through 64 plus 135 through 167) are indicated with circled numbers, a numbering system used also in Fig. 3. The system (2) numbers each tryptic peptide according to its sequential position in the hGH structure (cleavage of the disulfide bridges is assumed). The "X" denotes a citraconyl group. The amino acid sequence (8) of residues 20 through 64 is: Leu-His-Gln-Leu-Ala-Phe-Asp-Thr-Tyr-Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-Leu-Gln-Asn-Pro-Gln-Thr-Ser-Leu-Cys-Phe-Ser-Glu-Ser-Ile-Pro-Thr-Pro-Ser-Asn-Arg.

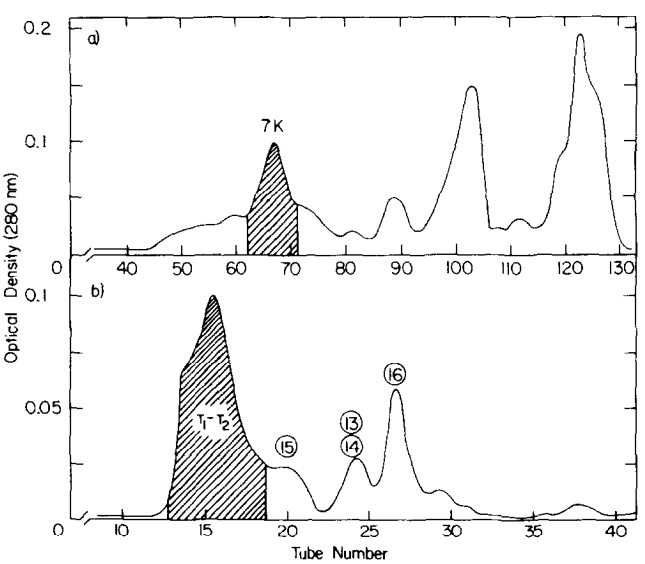

Fig. 2. *a: Separation of the tryptic peptides of citraconylated 20K on a column of Sephadex G-50 Superfine.* The location of the core peptide of 20K is designated by the shaded area (7K). *b: Separation of the tryptic peptides of the performic acid oxidized core peptide (7K) of 20K on a column of Sephadex G-25 Superfine.* The T_1-T_2 area was used for further characterization (Fig. 3*c*). The circled numbers denote peptides shown in Fig. 1.

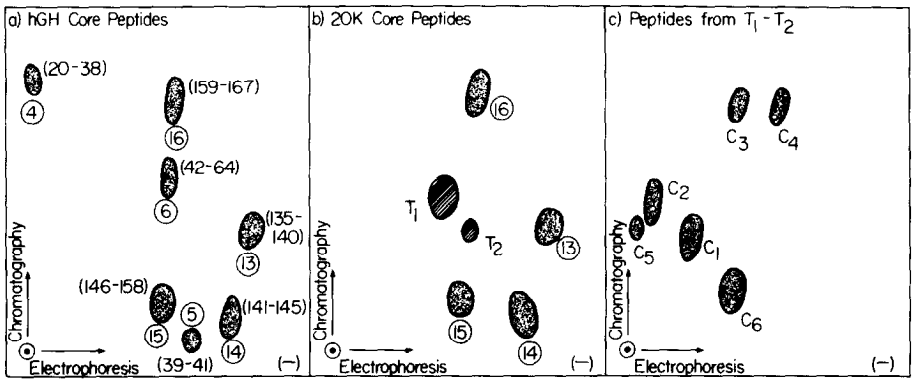

Fig. 3. *a* and *b*: *Tryptic peptide maps of oxidized core peptides of hGH and 20K.* The circled numbers indicate the peptides shown in Fig. 1; those in parentheses show the sequence numbers of each peptide. *c*: *Chymotryptic peptide map of the* T_1-T_2 *peak of Fig. 2b.* Compositions are given in the text.

peptide from hGH dissociated when reduced to a 5,500-dalton and a 3,500-dalton peptide. Molecular weight values are not very accurate in this region but relative sizes and dissociation products were valuable in identifying the core peptides

Structure analysis of tryptic peptides. That we had the correct core peptides was confirmed by the amino acid sequence of the first 6 residues of the two chains of the 9K and 7K peptides. In each case the sequences of 20 through 25 and 135 through 140 were found.

Fig. 3*a* and *b* show the peptide maps obtained with tryptic digests (lysines unblocked) of the oxidized core peptides. The map of the hGH peptides (Fig. 3*a*) was as expected from previous work (2). At that time we were unable to locate peptide 4 in a digest of the entire protein, but the peptide, residues 20 through 38, was easily detected in the core peptide.

The map of the core peptide of 20K (Fig. 3*b*) differed from that of hGH by not having peptides 4, 5 and 6, and instead, the major peptide T_1 and a minor peptide T_2 were seen. Analyses showed the composition of T_1 to be $Cys(SO_3)_{1.0}$ $Asx_{2.6}$ $Thr_{2.3}$ $Ser_{3.7}$ $Glx_{4.6}$ $Pro_{2.4}$ $Ala_{1.5}$ $Ile_{1.2}$ $Leu_{3.0}$ $Tyr_{1.0}$ $Phe_{2.5}$ $His_{1.4}$ $Arg_{0.7}$. When sequenced, peptide T_1 gave the following partial structure: (Leu)-His-Gln-(Leu)-Ala-Phe-Asp-Thr-Tyr-Gln-Glu-Phe-Asn-Pro-. After that, the analysis became uncertain. The method of Chang *et al.* (7) does not distinguish between

Leu and Ile. This sequence and the hGH structure beginning at position 20 are identical for the first 12 residues but then the 20K sequence of Asn-Pro is not found in hGH until positions 47 and 48 (see legend of Fig. 1 for amino acid sequence of hGH in this region). This was interpreted to mean that the 15 amino acid sequence of residues 32 through 46 is missing from 20K. With these 15 residues deleted from the sequence 20 through 64, the resulting peptide would have the composition of $Cys(SO_3)$ Asx_3 Thr_3 Ser_4 Glx_5 Pro_3 Ala Ile Leu_3 Tyr Phe_3 His Arg, a composition that agrees well with that found for peptide T_1. The other peptides of the 20K core, peptides 13 through 16 (Fig. 3*b*), were identified by amino acid composition. They were identical to those found in hGH (Fig. 3*a*).

We are not certain of the nature of the minor T_2 peptide (Fig. 3*b*); its amino acid composition was similar to T_1 but could not be considered identical. We had only enough peptide to determine the sequence of the first three residues, (Leu)-His-Gln, which was the same as for T_1.

Structure analysis of chymotryptic peptides. Chymotrypsin digestion of the T_1-T_2 material (Fig. 2*b*) produced a number of peptides (Fig. 3*c*). The amino acid composition of C_1 was $Cys(SO_3)_{0.8}$ $Asx_{2.2}$ $Thr_{1.5}$ $Ser_{3.9}$ $Glx_{3.4}$ $Pro_{2.8}$ $Ile_{0.9}$ $Leu_{0.8}$ $Phe_{1.6}$ $Arg_{1.0}$ and its partial sequence was Gln-Glu-Phe-Asn-Pro-Gln-Thr-Ser-(Leu)-( )-Phe-. Positive identification of additional residues was difficult. By referring to the amino acid sequence in the legend of Fig. 1, it can be seen that the sequence of C_1 begins at residue 29 of the hGH structure, continues for two more amino acids and then skips to position 47. The results again indicate a deletion of 15 amino acids, residues 32 through 46. With these 15 amino acids deleted, the peptide made up of residues 29 through 31 plus residues 47 through 64 would have the composition $Cys(SO_3)$ Asx_2 Thr_2 Ser_4 Glx_4 Pro_3 Ile Leu Phe_2 Arg. This is in good agreement with the composition found for peptide C_1.

Peptide C_2 gave the partial structure Asp-Glu-Phe-Asn-Pro-, the sequence found for C_1 except that the first residue was Asp instead of Gln. This suggests that there may be an allelic form of 20K and would explain the origin of peptide T_2 mentioned above.

The compositions of the other chymotryptic peptides (Fig. 3*c*) were as follows. C_3: $Asx_{1.3}$ $Thr_{1.1}$ $Glx_{1.3}$ $Ala_{0.9}$ $Leu_{1.8}$ $Tyr_{1.0}$ $Phe_{1.0}$ $His_{1.0}$ (residues 20 through 28); C_4: $Glx_{1.3}$ $Ala_{0.9}$ $Leu_{1.8}$ $Phe_{1.1}$ $His_{1.0}$ (residues 20 through 25); C_5: $Asx_{0.9}$ $Thr_{1.0}$ $Tyr_{1.1}$ (residues 26 through 28); and C_6: $Asx_{0.9}$ $Thr_{0.7}$ $Ser_{2.1}$ $Glx_{1.0}$ $Pro_{1.6}$ $Ile_{0.6}$ $Arg_{0.8}$ (residues 55 through 64).

DISCUSSION

The results reported here suggest that 20K is the product of a deletion mutation in the human growth hormone gene. The question to be answered now is whether 20K is a separate pituitary hormone with its own metabolic role. Nearly 50 individual human pituitary glands have been examined and the variant was found in each. This is strong indication that 20K has a ubiquitous distribution. Furthermore, 20K is the second most abundant hormone in the pituitary gland amounting to approximately 15% of the growth hormone. The 22,000-dalton form of growth hormone is the most prevalent.

Niall *et al.* (9) pointed out four internally homologous sequences in growth hormone, the first of which ends at approximately residue 32. The area of deletion in 20K starts at residue 32, falling, therefore, within an area of high variability.

ACKNOWLEDGMENTS

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