

A new immunoglobulin variant: $\gamma 3$ heavy chain disease protein CHI

(deletion/heterogeneity/extra fragment/tandem duplications/unequal crossover)

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ABSTRACT Protein CHI is a defective human $\gamma 3$ heavy chain immunoglobulin with a deletion encompassing a portion of the variable and constant regions. Joining of the two pieces takes place at the beginning of an extra fragment (Fh) in the constant region where repetitive sequences are found, apparently as the result of gene duplications and/or unequal crossover between γ genes. It is postulated that a 45 nucleotide fragment is the repetitive unit coding for the extra fragment.

It is generally accepted that polypeptide chains of immunoglobulin (Ig) are coded by two genes (*V*: variable and *C*: constant) (1). The joining mechanism is not known although a number of theories have been put forward (2-4). Deletion mutants of human and mouse myeloma heavy (H) chains (5, 6) have shown that joining between shorter *V* and *C* genes can take place at sites different from the normal switch region which is located around position 120 from the amino terminus of the H chain (7). Predominant among these unusual sites is position 216 ($\gamma 1$ numbering) (8), the beginning of a very intriguing section of the H chain containing the S-S bonds linking the two H chains (hinge region). This region can undergo short duplications as was shown for α chains (9) and a defective $\gamma 3$ Ig H chain (10). In the latter case the nature of the duplications was difficult to ascertain since the protein lacked the entire *V* region and part of the *C*; hence, the molecule might be either a gene product or a proteolytic fragment of a precursor (10).

The present study carried out on an unusual $\gamma 3$ mutant, protein CHI, indicates that this defective molecule is the product of both the *V* and *C* genes and therefore more suitable for defining the genetic defect and the nature of the duplicated fragment.

METHODS

Isolation of Protein CHI. The protein was obtained from a patient (11) with heavy chain disease (HCD) (12, 13) and purified by starch zone electrophoresis (14). Immunoelectrophoresis and Ouchterlony double diffusion in agar (15) were performed with antisera to γ chains, Fd, Fab and Fc fragments, and light (L) chains.

Physicochemical Studies. Molecular weight (M_r) determinations were performed in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (16). M_r 's of cyanogen bromide (CNBr) fragments were measured either by filtration on Sephadex G-50 (17) or by equilibrium sedimentation (18). Reduction was with 5 mM dithiothreitol and alkylation with iodo[^{14}C]acetic acid (0.7 Ci/mol) (19). Digestion with CNBr was carried out overnight at room temperature with a fivefold excess (wt/wt) of CNBr to protein in 70% formic acid. Reduced and alkylated CNBr fragments were separated on a 200×4 cm Sephadex G-75 column in 5% formic acid.

Chemical typing and tryptic fingerprints were performed as described (19, 20).

Abbreviations: HCD, heavy chain disease; CNBr, cyanogen bromide; M_r , molecular weight.

Enzymatic Digestion. Digestion by pepsin (Worthington, twice crystallized) was performed at an enzyme/substrate ratio of 1:50 (wt/wt) in 5% formic acid for 16 hr at 37°, and by L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (Worthington) in 0.2 M NH_4HCO_3 , pH 8.2 for 6 hr at 37° at an enzyme to substrate ratio of 1:50 (wt/wt). Digestion by Pronase (Sigma Chemical Co.) of completely reduced protein and fragment IV (21) was done in 0.1 M NH_4HCO_3 , pH 8.2, for 3 hr at 37° at an enzyme to substrate ratio of 1:50 (wt/wt). Digestion by carboxypeptidase A and B (Worthington) was done in 0.1 M NH_4HCO_3 for 2 and 8 hr at 37° with 5 μg of enzyme per 0.01 μmol of peptide.

Purification of peptides, amino acid analysis and determination of NH_2 -terminus and sequence were done as described (22). Automatic amino acid sequencing was performed with a Beckman model 890C sequencer by the method of Edman and Begg (23). Released derivatives were identified by gas chromatography with a Hewlett-Packard gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.), by amino acid analysis of the hydrolyzed derivatives, and by thin-layer chromatography (24).

RESULTS

Native protein CHI had a M_r of 80,000 and subunit of 44,000 after reduction and alkylation. It strongly precipitated with antisera to Fc but not with antisera to L chains, or to Fab and Fd fragments. Chemical typing of the partially reduced and alkylated protein showed it to be of the $\gamma 3$ subclass of IgG. Fingerprint analysis indicated that the Fc fragment was intact and contained the peptides characteristic for the hinge region and carboxyl end of $\gamma 3$ as will be described elsewhere.

Isolation and characterization of a CNBr fragment

Purified protein (300 mg) was partially reduced and [^{14}C]car-

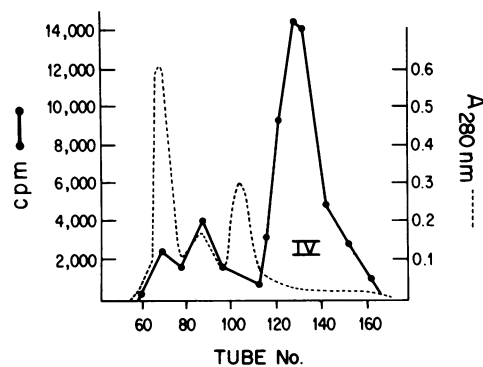


FIG. 1. Separation of CNBr fragments of protein CHI. The partially reduced and [^{14}C]carboxymethylated fragments were applied on a Sephadex G-75 column equilibrated with 5% formic acid (200×4 cm). Fractions of 6 ml were collected, and the flow rate was 25 ml/hr. The fourth peak contained the amino terminal of the molecule and the inter-heavy disulfide bonds.

Table 1. Amino acid composition* of a CNBr fragment obtained from protein CHI (peak IV, Fig. 1) and its tryptic (T) and peptic (P) peptides

Peptide	T1	T2	T3	T4	T5	T6	T1a	Peak IV	P1	P2	P3	P4	P5
Lys	2.2		1.0		2.1		0.7	7.7		1.1	4.2		2.0
His		0.7						0.9			0.9		
Arg		0.8		1.0				3.7			4.0		
CMCys†		0.6	1.0	1.3	0.8			7.1			6.0		
Asp	0.9	1.0		1.1		0.9	0.8	7.1	1.0		4.1		1.1
Thr	1.0	3.5		0.9		0.8	0.8	8.6		0.9	6.6		0.9
Ser	4.0			1.1	1.0		1.2	9.2		3.3	3.1	1.1	
Glu	4.3		1.1		1.1		1.2	9.3	2.0	2.1	4.1		
Pro	2.0	1.5	1.9	3.5	5.0		1.0	24.6		1.8	20.0	1.0	2.5
Gly	2.6	1.0			2.1		1.0	6.7		3.1	1.1	2.1	
Ala	0.7				1.0		0.8	2.7		0.8	0.8		
Val	0.6				0.8		1.3	2.6	0.8			0.8	
Met‡						0.8		0.8					0.8
Leu	4.0	1.0			3.2	1.0	0.9	9.7	1.1	1.8	3.0	1.0	2.1
Phe					1.8			2.0				1.0	1.0
CHO							+	+					
N-terminus	PCA	Thr	CMCys	Ser	CMCys	Asp	Neg	PCA	PCA	Glu	Leu	Leu	Leu

CHO, carbohydrate; PCA, pyrrolidone carboxylic acid; Neg, negative.
 * Compositions are reported as mol of amino acid per mol of peptide. The data for peak IV are based on a M_r of 12,600-hydrolysis for 21 hr.
 † Determined as carboxymethylcysteine.
 ‡ Determined as homoserine lactone.

boxymethylated. After treatment with CNBr, the fragments were separated on Sephadex G-75 in 5% formic acid (Fig. 1). The intact molecule and CNBr fragment IV had an unreactive amino-terminus. Digestion of both by Pronase released three related ninhydrin-negative chlorine-positive peptides (PCA, Val; PCA, Val, Glu; and PCA, Val, Glu, Leu). Peak IV therefore contained the amino terminal end of protein CHI and isolation of radioactive peptic-tryptic peptides (19) indicated that peak IV also contained the inter-heavy chain disulfide bridges of the molecule. Only peak IV will be discussed in this paper. The M_r of peak IV was determined by equilibrium sedimentation in 0.15 M potassium chloride, 5 M guanidine hydrochloride, and 0.01 M -(N-morpholino)ethanesulfonic acid (MES) at pH 7.0. The weight average M_r in both solvents was 12,600. (The partial specific volume was assumed to be 0.72.) Tryptic fingerprinting of peak IV gave seven peptides less than would be expected

from the Lys and Arg content (Table 1), suggesting that some of them were duplicated as shown for another defective $\gamma 3$ protein (10). All tryptic peptides and some peptic peptides were then isolated from peak IV. Their amino acid compositions and sequences are shown in Table 1 and Fig. 2. Digestion with pepsin gave five major peptides and free serine. The partial sequence of peptide P3 established the overlaps between T1-T2 and T3-T4. It was not possible to obtain an overlap between T4 and T5 since the yield dropped after position 20. Moreover, the proline residue was carried over after position 24. Peptide P5 established the overlap between T5 and T6. The amino acid composition of peptide P3 (M_r :7500) which resembles that of a chymotryptic peptide obtained from an intact myeloma protein of the same subclass indicates that it contains the duplicated fragments (25). The data fit well if a similar or identical sequence is repeated three times as indicated in Fig. 2. The

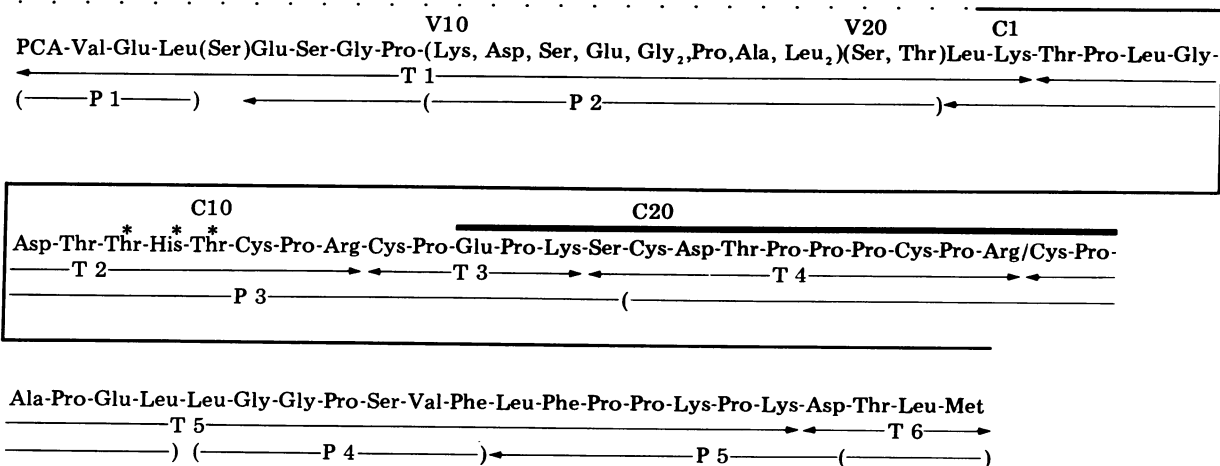


FIG. 2. Amino acid sequence of tryptic (T) and peptic (P) peptides from peak IV (Fig. 1). Digestion of peptide T1 by carboxypeptidase B and A released Lys (1.0), Leu (0.80) and equal amounts of Ser and Thr (0.50). (Ser) uncertain: obtained as free amino acid; P1 was also isolated after Pronase digestion (see text). The partial sequence of P3 was determined by automatic sequencer. / indicates overlap is not available. Thick line indicates the postulated repetitive fragment. *Homology with the hinge region of IgG1. . . (V) variable region. — (C) constant region. The site of the beginning of C region, in this case the Fh fragment (see Fig. 3), is tentative. Complete amino acid sequence of an intact $\gamma 3$ H chain is not available.

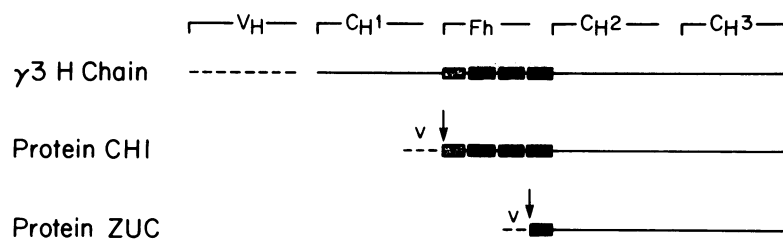


FIG. 3. Comparison of the deletions in defective human $\gamma 3$ proteins. On top are represented the suggested domains of a normal $\gamma 3$ heavy chain: - - - variable; — constant. Fh section containing an extended hinge region with similar \square or \blacksquare identical duplications. Arrows indicate the two different sites where normal sequence begins after internal deletions in proteins CHI (this paper) and ZUC (31).

expected eleven cysteine residues were not found due to low recovery of carboxymethylcysteine (Table 1). Consequently until the complete sequence is available this arrangement remains tentative. Peak IV contained a low yield tryptic carbohydrate-containing peptide (Table 1, peptide T1a). Its origin is not clear since it does not seem to be derived from T1 and it is different from another carbohydrate-containing peptide from the Fc fragment (Asp, Thr, Ser, Glu₃, Phe₂, Arg).

DISCUSSION

Elucidation of the complete amino acid sequence of an $\gamma 1$ chain (8) demonstrated that the region containing the interchain bridges (hinge or interdomain region) which lies between the C_{H1} and C_{H2} domains, does not have homology with the rest of the chain. In the other subclasses of IgG the sizes of the hinge vary, as do the number of cysteine residues and their positions (19).

Particular attention has been focused on the $\gamma 3$ H chain after it was shown that an extra fragment of 8–10,000 daltons (Fh fragment) (26), consisting of duplications (10) of the previously reported hinge region of IgG3 containing five cysteine residues (22) was located between C_{H1} and C_{H2} domains. However, the number and nature of the repetitive sequences were not clearly defined. The availability of an unusual $\gamma 3$ HCD protein CHI offered the opportunity to do so. The M_r of protein CHI (monomer) was 44,000 instead of 58–60,000 reported for normal $\gamma 3$ chains (27, 28). A CNBr fragment was obtained which contained the amino-terminus of the molecule and the above mentioned extra fragment. Its M_r was 12,600. Amino acid analysis (Table 1), fingerprints, and studies of amino acid sequence (Fig. 2) indicated that after approximately 22 residues which belong to the amino terminus of the V region, as determined by homology to other human V_H regions (7), there is a gap of about 200 amino acids, comprising the remainder of the V region, the switch region and part of the C gene (C_{H1} domain). Normal sequence resumed at the beginning of the Fh fragment, and in the absence of knowledge of the sequence of C_{H1} of $\gamma 3$ chain the joining point between V and C is tentatively placed around position 23, Fig. 2. The presence of peptide T1a in peak IV (Table 1) is intriguing; it was chlorine-positive and had an unreactive amino terminus. Since it has carbohydrate it could be due to an unusual tryptic splitting of peptide T1, which is the amino terminal peptide of the molecule and belongs to the V region. However, its amino acid composition does not fit either at the amino or carboxyl terminal of T1. Furthermore, peptide T1 does not contain carbohydrate. The possibility exists that peptide T1a is another peptide different from T1 and located between T1 and T2 (Fig. 2) or more probably at the amino-terminal end. The latter may also be true in protein CRA (unpublished observations) where heterogeneity was observed at the amino-terminus (29) and it suggests that two variable peptides coming from two different genes have

joined the same C gene, or alternatively a mutation of the V gene.

The Fh fragment appears to have 60–65 residues and a section of 15 residues is repeated three times as shown in Fig. 2. Based on M_r determinations of peak IV and peptide P3 and their amino acid compositions (Table 1), the repetitive fragment should be contained in P3. The beginning of the duplicated segment can be placed between position 11 to 16 of the Fh fragment without an altered interpretation. However, glutamic acid at position 16 of the Fh fragment (which corresponds to position 216 in a normal $\gamma 1$ chain) (8) appears more attractive in light of its role as a genetic hot spot, which internal deletions end or start in defective H chains in humans (5, 30) and mice (6). The beginning of the Fh fragment can be considered as another duplication although it is not identical to the others. In fact positions 8, 9, and 10 marked by asterisk in Fig. 2, are more like the $\gamma 1$ hinge (8) than the $\gamma 3$ hinge, and suggests that unequal crossing over may have taken place between two different γ genes, although independent mutations of the $\gamma 1$ and $\gamma 3$ genes should also be considered.

Whether the number of postulated duplications applies to an intact $\gamma 3$ H chain remains an open question. Comparison with ZUC (Fig. 3), the first internally deleted $\gamma 3$ HCD protein reported (31), shows that in the case of variant ZUC, a hinge peptide containing fifteen residues is present but not its duplications. These findings raise the possibility that in internally deleted $\gamma 3$ genes, the joining at the DNA level between defective V and C genes is not a random process but can occur at any one of its tandem repeats. It seems possible that the Fh fragment of $\gamma 3$ chain arose by gene duplication from a DNA fragment 45 nucleotides in length.

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