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Protein Hal: Partial Deletion of a "γ" Immunoglobulin Gene(s) and Apparent Reinitiation at an Internal AUG Codon

(heavy-chain disease/aminoacid sequence/internal deletion/methionine 252)

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ABSTRACT **Protein Hal is a human γ4 heavy-chain disease protein whose molecular weight is reduced from 55,000 to 25,000 in 6 M guanidine due to the lack of disulfide bonds between heavy chains. Studies of aminoacid sequence indicate that it contains a gap of about 240 residues, starting 10 residues from the N-terminal end and including the rest of the Fd fragment, as well as the hinge region. Normal sequence apparently resumes at a methionine residue (position 252) in the second constant domain (C_H2) and seems normal from there to the carboxyl end of the molecule. These results imply that reinitiation of translation at an internal AUG codon occurs in protein Hal.**

Gamma heavy-chain disease (γHCD) is characterized by the presence in serum and urine of a protein related structurally to the Fc fragment of immunoglobulin heavy chains of humans (1). Interest in these natural fragments was stimulated when it was shown that the γHCD proteins were not extracellular degradation products, but rather the result of abnormalities of gene expression. Thus, if more than one gene was involved in the synthesis of a single heavy chain, then the HCD proteins could result from a mutation affecting only one of them. Detailed structural studies (2–4) on three γHCD proteins have shown the defect to be an internal deletion of part of the Fd fragment, with resumption of normal sequence at the glutamic acid residues present at position 216 (the numbering sequence is appropriate for the γ1 heavy chain). This point marks the beginning of the hinge region, a section of the heavy chain between C_H1 and C_H2 domains (5), which contains the interheavy-chain disulfide bonds and is unique in not having a homologous counterpart in the remainder of the molecule (6, 7). These data suggested the possibility that the constant (C) region of immunoglobulin heavy chain was under the control of more than one gene and that position 216 could mark the beginning of another cistron (3). Although other interpretations are also possible (8–10), the finding of protein Mcg (11), a myeloma protein with a deletion of a portion of the hinge region only, further emphasized the importance of position 216. The sequence of Mcg heavy chain shows that it is normal through the first 215 residues and has a gap of 15 amino acids that commences at position 216.

Abbreviations: Nomenclature of immunoglobulins and their chains and fragments follows the recommendation of World Health Organization [*Bull. WHO* **30,** 447 (1964); **33,** 721 (1965); **35,** 953 (1966); **38,** 151 (1968)]. Hsr, homoserine; HCD, heavy chain disease.

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We present in this paper, studies on a new type of γHCD protein. Protein Hal is a γ4 HCD protein with a different deletion: the gap encompasses almost the entire Fd fragment as well as the hinge region. Reinitiation of synthesis seems to occur at a methionine residue in position 252.

MATERIALS AND METHODS

Isolation of the Hal Protein. The source of the Hal protein was a patient with malignant lymphoma accompanied by the presence in serum, urine, and pleural and peritoneal fluids of a gamma heavy-chain fragment belonging to the IgG4 subclass (12). All studies were performed on pleural or peritoneal fluid collected on ice and cleared of cellular debris by centrifugation before storage at −20°.

Pleural fluid was adjusted to 20% saturation with ammonium sulfate. The resulting precipitate was removed by centrifugation at 4000 × *g*, and the supernatant was adjusted to 40% saturation with ammonium sulfate. The second precipitate was collected after centrifugation, dissolved in water, and dialyzed against phosphate (0.01 M)-buffered saline (0.15 M), pH 7.4. After dialysis, the solution was again adjusted to 40% saturation with $(NH_4)_2SO_4$. The precipitate was recovered, dissolved in water, and dialyzed against 0.05 M sodium acetate buffer, pH 5.5. The protein solution was applied to a carboxymethyl-Sephadex (CM-50) column; elution was performed with acetate buffer. The elution profile consisted of a single peak with a trailing "shoulder." Fractions constituting the peak of protein concentration were combined, lyophilized, dissolved in 4.0 M guanidine hydrochloride, and applied to a Sephadex G-100 column equilibrated with this solvent. The elution profile again consisted of a single peak; this purification step removed noncovalently-linked carbohydrate from the Hal protein.

Ultracentrifugation. Molecular weights were determined by the high-speed meniscus depletion method (13) in a Beckman model E analytical ultracentrifuge equipped with interference optics. Weight average and number average molecular weights were calculated at regular intervals throughout the cell with an IBM system 360 computer and a program developed by D. A. Yphantis and D. E. Roark (personal communication). The value of $\bar{v}$ (partial specific volume) was determined pycnometrically; this value was 0.689 in guanidine·HCl and 0.712 in phosphate-buffered saline.

Disc Gel Electrophoresis was performed in 10 M urea in 10% acrylamide gels (14); the Hal protein was also examined after treatment with sodium dodecyl sulfate (15).

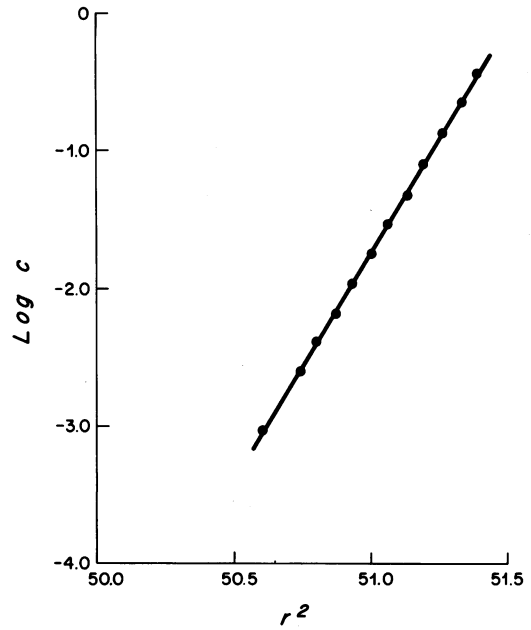

FIG. 1. Plot of log concentration (c) against r^2 for γHCD protein Hal. The sample examined was isolated as described in *Methods* and exhaustively dialyzed against 6 M guanidine·HCl + 0.1 M dithiothreitol. The speed of the centrifuge was 52,000 rpm and the temperature 20°.

Selective Reduction and Radioactive Labeling of Interchain Bridges ("Chemical Typing"). 5 mg of Hal protein was dissolved in 0.5 ml of Tris·HCl buffer (0.5 M, pH 8.2) and partially reduced; the interchain bridges were specifically labeled with [^{14}C]iodoacetic acid. The protein solution was dialyzed against 5% formic acid for peptic digestion. Details of the methods have been described (16, 17).

Total Reduction and Alkylation with [^{14}C]Iodoacetic Acid. The intact protein (50 mg) and fragments produced by cleavage with cyanogen bromide (CNBr) were totally reduced as follows: the proteins were dissolved in 6 M guanidine hydrochloride–0.5 M Tris·HCl buffer, pH 8.6, at a concentration of 20 mg/ml and treated with 50 μl of a 10 mg/ml solution of dithiothreitol. Reduction was performed for 1 hr at room temperature (25°) under N_2. 100 μl of 0.1 M [^{14}C]iodoacetic acid (0.5 Ci/mol) was added, and the mixture reacted for 1 hr at room temperature. A Sephadex G-25 column eluted with 0.3 M acetic acid was used for desalting.

Enzymatic Digestion. Pepsin (Worthington, twice crystallized) digestion was performed at an enzyme/substrate ratio of 1:50 (w/w) in 5% formic acid for 16 hr at 37°, and with trypsin (treated with L-1-tosyl-amido-*w*-phenyl-ethyl-chloromethyl ketone, Worthington) at a ratio of 1:50 in 0.2 M NH_4HCO_3, pH 8.3, for 6 hr at 37°.

Pronase (Sigma Chemical Co.) digestion of completely reduced Hal protein was done in 0.1 M NH_4HCO_3, pH 8.2, for 3 hr at 37° at an enzyme/substrate ratio of 1:50 (w/w). Subtilisin (Nutritional Biochemical Co.) digestion of completely reduced protein was done in 0.1 M NH_4HCO_3, pH 8.2, for 6 hr at 37° at an enzyme/substrate ratio of 1:50(w/w).

Carboxypeptidase A (Worthington, diisopropylphosphorofluoridate-treated) digestion was performed in 0.1 M NH_4HCO_3 for 4, 6, and 16 hr at 37° with 5 μg of enzyme per 0.01 μmol of peptide.

Radioactive Peptides Were Purified by high-voltage electrophoresis on Whatman No. 3MM paper at pH 3.5. The peptides were then subjected to high-voltage electrophoresis at pH 6.5 and 2.1 (18). Electrophoresis was done for 60 min. The following buffers were used for separation: at pH 3.5, pyridine–acetic acid–water 1:10:190 (v/v); at pH 6.5, pyridine–acetic acid–water 33:1:300 (v/v); and at pH 2.1, formic acid–acetic acid–water 1:4:45 (v/v). The radioactive peptides were localized by autoradiography with Kodak Royal Blue Medical x-ray film. Mobilities at pH 6.5 are expressed as fractions of the distance between ε-Dnp-lysine and aspartic acid; amide residues were assigned on the basis of electrophoretic mobilities of peptides at pH 6.5 (19). Acid hydrolysis, aminoacid analysis, and N-terminal and sequence determinations were done as described (18). Ambiguities between ε-(DNS)-lysine, α-DNS-histidine, and DNS-arginine were resolved by further electrophoretic separations in the second dimension with 50 mM trisodium phosphate–ethanol 3:1 (v/v) (20).

Cystine Diagonal Maps. This procedure was performed at pH 3.5 by the method of Brown and Hartley (21) on peptic–tryptic digests of Hal protein.

Cyanogen Bromide (CNBr) Cleavage. Hal protein (50 mg) in 2.5 ml of aqueous 70% formic acid was treated with 150 mg of CNBr for 25 hr at room temperature with stirring. The digest was diluted 20-fold, freeze dried, and applied to a Sephadex G-100 column (140 × 3.5 cm) in 1 N acetic acid. Absorbance was measured in a Beckman DU spectrophotometer at 280 nm. Fragments were characterized by identification of their N-terminal residues, aminoacid analysis, and isolation of carboxymethylcysteine-containing peptides after total reduction and alkylation. Radioactivity was counted in a Beckman 150 scintillation counter.

RESULTS

Physical Characteristics of Hal Protein. The molecular weight of Hal protein was determined by high-speed equilibrium ultracentrifugation. The weight average molecular weight in aqueous buffers, extrapolated to infinite dilution, was 55,000. In 6 M guanidine·HCl, the weight average molecular weight extrapolated to infinite dilution was 25,000, and in 6 M guanidine·HCl plus 0.1 M dithiothreitol the molecular weight was 25,073. In Fig. 1, the linearity of the plot indicates that the protein is homogeneous with respect to molecular weight.

On acrylamide gel-electrophoresis of isolated Hal protein in either urea or sodium dodecyl sulfate, a single band of protein was observed. These findings again indicate that the protein is homogeneous both with respect to charge and to molecular size. Samples electrophoresed in the presence and absence of dithiothreitol had the same mobility, confirming the absence of interchain disulfide linkages.

Position and Function of Half-Cysteine Residues. "Chemical typing" has been used successfully in other proteins (16, 17) to detect hinge-region peptides that contain the interheavy-chain disulfide bonds. Protein Hal was partially reduced, alkylated with [^{14}C]iodoacetic acid, and subjected to peptic and tryptic digestion. The absence of any radioactive peptides suggested that the Hal protein lacks the hinge-region peptide. No free SH groups were present in protein Hal,

since the molecule did not incorporate [^{14}C]iodoacetic acid without previous reduction in the presence of 6 M guanidine.

In contrast, after complete reduction and alkylation with [^{14}C]iodoacetic acid, digestion with pepsin–trypsin, and separation by electrophoresis at pH 3.5, four radioactive bands were seen. These bands correspond to bands obtained by similar treatment of an intact heavy chain from IgG4 myeloma protein, Wur (Fig. 2). The partial aminoacid sequence of the purified carboxymethylcysteine peptides is shown in Table 1. By comparison with proteins Eu (5) and Vin (22), the sequences found in the Hal peptides suggest that the peptides originate in the Fc fragment. Diagonal map electrophoresis showed them to be bridged as indicated in Table 1.

Amino-Terminal Sequence. An amino-terminal residue could not be detected in Hal protein either by the dansyl-chloride method or the automated sequencer, suggesting the presence of a blocked NH_2-terminal. In some preparations, small amounts of Leu,Phe were detected, indicating heterogeneity at the amino-terminal probably due to proteolysis (see below). A Pronase and a subtilisin digest of completely reduced and alkylated Hal protein was passed through a Dowex (AG 50W-X2) column, and eluted peptides were purified by paper electrophoresis at pH 6.5 (23). The aminoacid analyses of these peptides are shown in Table 2 and correspond to the NH_2-terminal peptides of the H chain. A carbohydrate-containing peptide was also obtained and its sequence, Asn-Ser-Thr, suggests that it corresponds to position 297 (5).

Isolation of CNBr Fragments. Protein Hal, which contains three methionine residues (Table 3), was treated with CNBr, and the fragments were separated on Sephadex G-100 in 1 N acetic acid (Fig. 3). As expected, four peaks were obtained. Peaks III and IV showed little absorbance at 280 nm; the fragments comprising these peaks were further purified by paper electrophoresis at pH 3.5. The aminoacid composition and NH_2-terminal amino acid of CNBr fragments are shown in Table 3. Complete reduction and alkylation with [^{14}C]-iodoacetic acid of CNBr fragments showed radioactivity to be present only in peaks I and II. These peaks were digested with pepsin–trypsin, and their carboxymethyl-cysteine peptides were purified by paper electrophoresis. Cysteines 261 and 321 (Table 1) were present in peak I and cysteines 367 and 425 in peak II.

Peak III contained no homoserine and its NH_2 terminal was His. Trypsin digestion released a peptide whose sequence corresponds to that of the C-terminal sequences previously

TABLE 1. *Peptic–tryptic carboxymethylcysteine peptides obtained after complete reduction and alkylation of protein Hal*

Position*	Sequence†
261	Thr-Pro-Glu-Val-Thr-Cys-Val
321	Cys-Lys‡
367	Thr-Cys-Leu
425	Ser-Cys-Ser-Val-Met

* The *numbers* indicate the position corresponding to the sequence of Eu H chain (5). Cys-261 is bridged to 321, and 367 to 425 (see Text).

† These are identical to those in γ4 H chain Vin (6, 22).

‡ A related (more basic) peptide was also obtained in low yield; its sequence is Lys-Cys-Lys (see also Fig. 2).

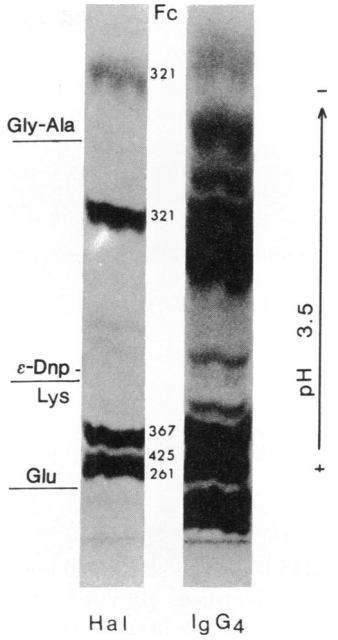

FIG. 2. Autoradiograph of the radioactive peptides from a peptic–tryptic digest of completely reduced and [^{14}C]iodo acetic acid-labeled γHCD protein Hal compared by electrophoresis at pH 3.5 with peptides similarly obtained from the heavy chain of an intact IgG4 myeloma protein, Wur. *Numbers* refer to the position of the peptides in the Fc fragment. Two peptides were obtained corresponding to position 321 (see Table 1).

reported for γ4 heavy chain (22, 24). The presence of Leu instead of Pro at the penultimate position is characteristic of the γ4 subclass.

Peptides constituting peak IV failed to stain with ninhydrin, had no reactive amino group, but did react with hypochlorite–iodine reagent. We concluded that peak IV contained the N-terminal fragment of Hal protein. Carboxypeptidase A released homoserine (Hsr) (1.0),Ser (1.0),Leu (0.8),Val (0.4). Taken together with the aminoacid composition of Pronase and subtilisin peptides (Table 2), this information suggests the partial sequence shown in Fig. 4. Insufficient material was available for further digestion and sequence determination.

In one instance, peak II had a heterogeneous NH_2-terminal; not only was Thr present (Table 3), but also small amounts of Leu,Phe (see above for similar findings on the intact Hal protein). However, isolation of carboxymethylcysteine peptides showed that Cys 321 was present in Peak II, indicating that under certain conditions, the molecule undergoes degradation that stops somewhere between Cys 261 and 321. Fig. 4 shows

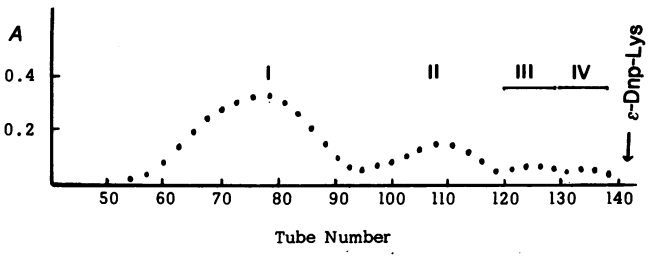

FIG. 3. Separation of CNBr fragments of protein Hal on a Sephadex G-100 column equilibrated with 1 N acetic acid. Absorbance of effluent at 280 nm.

	252
Peak IV	Pyrrolidone carboxylic acid (Glu,Thr) (Glu_2,Asp,Gly)Val-Leu-Ser-Met
	Carbohydrate
	253 261 297 321 358
Peak I	Ile-Ser-Arg-Thr-Pro-Glu-Val-Thr-Cys-Val/Asn-Ser-Thr/Cys-Lys/ /Met
	359 367 425 428
Peak II	Thr-Lys/ /Thr-Cys-Leu/ /Ser-Cys-Ser-Val-Met
	429 446
Peak III	His (Glu,Ala,Leu,His,Asp,His,Tyr,Thr,Glu)Lys-Ser-Leu-Ser-Leu-Ser-Leu-Gly

FIG. 4. Partial sequence of CNBr fragments of γHCD protein Hal. For peaks I, II, III, and IV see Fig. 3 and Table 3. The *number* over the residue indicates the position corresponding to the sequence of Eu H chain (5). The first 10 residues in protein Hal come from the variable region (Hv). The first Met corresponds to position 252; from there on the sequence is identical to Vin, a γ4 chain (22).

the partial aminoacid sequence and alignment of CNBr fragments. We were unable to find any peptides other than those listed in Table 3.

DISCUSSION

Based on aminoacid sequence studies, three types of γHCD proteins have been identified. (Proteins resembling γHCD, but apparently arising on the basis of degradation after synthesis, are not included in this discussion; ref. 25 and unpublished findings.)

Type I: Proteins with partial deletion of the Fd fragment and resumption of normal synthesis at position 216. Three variants belong to this category: Zuc, Cra, Gif. In Zuc (2), a γ3 protein, there is a gap of almost the entire Fd fragment starting at residue 18. The Glu residue at position 19 of Zuc corresponds to position 216 (γ1 numbering) in the constant-region sequence found in γ3 heavy chains. From this point to the C-terminal end of the molecule, the Zuc γHCD protein appears to be normal. Cra (3), a γ1 HCD protein, contains a similar gap; after 10–11 residues there is a gap followed by a normal sequence that begins at the same residue as in Zuc. Gif (4), a γ2 HCD protein, has a gap of about 100–110 residues that starts at the end of the variable region and comprises the whole constant region of the Fd fragment. Reinitiation starts at the glutamic acid residue that is homologous to residue 216.

Type II: Deletion of part of the hinge region. So far, only one variant, Mcg (11), has been identified and assigned to this category. In this protein, the deletion includes 15 residues from the glutamic acid at position 216 to position 232.

Type III: Partial deletion of the Fd fragment and the hinge region. Proteins Hin (25, 26), Par (27), and Hal (28) belong to this category. The nature of the deletion has not been determined for the first two. Hal, described in this paper, is a γ4 HCD protein that dissociates in the absence of reducing agents and fails to show interheavy-chain bonds on "chemical typing." A short N-terminal section (10–11 residues) appears to be present, but the remainder of the Fd variable and Fd constant region is missing, since the characteristic intrachain disulfide bonds from this region and the heavy–light chain linkage section could not be demonstrated. The deletion includes about 240 residues, and includes the hinge region and the beginning of the C_H2 domain. Protein Hal therefore represents a noncovalently bound dimer of an internally deleted γ4 heavy chain. After the deletion, normal sequence appears to resume at an internal methionine residue, position 252, and from there on, the sequence is apparently normal. The possibility that reinitiation occurs before Met 252 cannot be excluded with certainty. However, this is an unlikely possibility, since in three experiments (see *Methods*) the results obtained by carboxypeptidase A digestion suggest that the sequence is Val-Leu-Ser-Met (Fig. 4, peak IV), while the sequence before residue 252 in all human γ-chain subclasses is Asp-Thr-Leu-Met (refs. 2, 5, and 22, and unpublished results). For evaluation of the significance of the present findings, other Type III γ-HCD proteins will have to be examined

TABLE 2. *N-Terminal peptides obtained from protein Hal*

Pronase	Pyrrolidone carboxylic acid (Glu, Thr)
Subtilisin	Pyrrolidone carboxylic acid (Glu_3, Thr, Asp, Gly)

TABLE 3. *Aminoacid analyses* of protein Hal and its CNBr fragments*

	Whole molecule†	CNBr fragments‡			
		I	II	III	IV
Lys	13	8	4.2	1	
His	5	1.2	1	3	
Arg	5	4	1		
Cys§	3.7	2	2		
Asp	19.5	10	7	1	1
Thr	16	7	7	1	0.7
Ser	21	10	7.4	3	1.2
Glu	30	16	8	2	4
Pro	13	8	5		
Gly	14	5	5.6	1	0.8
Ala	6	2.2	2.5	1	
Val	16	10	4		0.6
Met¶	3	+	+		+
Ile	4.5	3	1.1		
Leu	16.3	6	5.1	4	1
Tyr	7	4	2.2	1	
Phe	7	3	3.2		
Trp	N.D.‖				
CHO	+	+			+
NH_2-terminal	PCA	Ile	Thr	His	PCA

* 20 hr hydrolysis (not corrected).

† Residues per 200 found.

‡ Values reported are expressed as residues per mol.

§ Determined as carboxymethylcysteine or cysteic acid.

¶ Methionine sulphone or (+) the sum of homoserine and homoserine lactone.

‖ N.D. = not done.

to determine whether common or unique reinitiation sites occur in these proteins.

The finding of Met as the likely site of reinitiation in Hal protein is of particular interest, since codon AUG functions as an initiation signal in protein biosynthesis in prokaryotes and possibly in eukaryotes (29, 30); when AUG occurs internally, it codes for insertion of methionine. Internal initiation sites have been described, but only in the presence of a nearby terminator triplet (31). If the same mechanism is involved in variant Hal, it seems likely that the amber codon must have occurred at the beginning of the V_H gene, thus allowing AUG, which codes for Met 252, to be used as a site for reinitiation.

In the rII-B cistron of bacteriophage T4 (32), both the nonsense fragment and a C-terminal fragment of the rII-B gene product are synthesized and detected separately. In protein Hal and the other HCD proteins, the two fragments are not only synthesized, but also peptide-bonded. Since such linkage is an unlikely result of normal ribosome function, a special joining enzyme would have to be postulated. Furthermore, for protein Hal, the internal Met, which appears to represent the reinitiation site, is not split off. The unlikely possibility remains that the currently recognized HCD proteins are exceptions, and that in other neoplastic diseases of lymphoid tissue both fragments are synthesized, released separately from the cell, and, perhaps, rapidly degraded. In any case, a careful search for such fragments seems to be warranted. In this respect, it is interesting to point out that a urinary protein (β2-microglobulin) present in normal individuals is related to the Fc fragment of IgG (33). The authors of this report postulated that the gene coding for the microglobulin may have evolved from an immunoglobulin gene by the use of a new start signal (Met 252) for initiating synthesis of the polypeptide, although some other interpretations are also possible (34). Whether the AUG codon at Met 252, or other codons different from AUG, also functions as an initiation site in the wild-type "γ" gene(s) is still an open question.

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