Molecular Defect in a Gamma-2 ($\gamma 2$) Heavy Chain

Abstract. *The first gamma-2 ($\gamma 2$) heavy chain disease protein Gif has pyrrolidinecarboxylic acid as its amino terminal residue, much of the Fd variable region, and an internal deletion of the heavy chain of about 100 residues corresponding to most of the Fd constant region. Normal sequence resumes with a glutamic acid residue at position 216 in the hinge region. This is the third gamma heavy chain disease protein where normal sequence resumes at the same position after the deletion.*

Gamma heavy chain disease (γHCD) (*1*) is characterized by the production of an aberrant protein which consists of the Fc fragment and part of the Fd fragment of immunoglobulin heavy chains (*2*). Investigations of three γHCD proteins have shown that the structural defect is an internal deletion. In protein Zuc (*3*), a $\gamma 3$ protein, there is a gap of almost the entire Fd fragment starting at residue 18. The Glu (*4*) at residue 19 of Zuc corresponds to position 216 (*5*) in the normal sequence found in $\gamma 3$ heavy chains, and from this point to the COOH-terminal end of the molecule it is apparently normal. Cra (*6*), a $\gamma 1$ HCD protein, has a heterogeneous NH_2-terminal region. After these 11 residues normal sequence is resumed at the same residue as in Zuc. In another HCD protein, Hi (*7*), there is also an internal deletion, but its size and exact location have not been identified. Studies of these proteins may yield valuable clues about the genetic control of immunoglobulin heavy chains.

Protein Gif was isolated from the serum by starch-block electrophoresis and repeated filtration on Sephadex G-200. It was typed as a $\gamma 2$ protein immunologically and chemically (*8*). Molecular weights were determined by acrylamide-gel electrophoresis in sodium dodecyl sulfate (*9*). The native protein exists as a dimer with a molecular weight of 80,000; the monomer formed by reduction has a molecular weight of 40,000.

Amino terminal analysis with the dansyl (diaminonaphthylesulfonyl) chloride method was negative, indicating a blocked NH_2-terminal. A Pronase digest of Gif was passed through a Dowex-50 column and then subjected to high-voltage electrophoresis at *p*H 6.5. A ninhydrin-negative, chloride-positive spot was identified as a dipeptide, Glu-Val. Tryptic peptide patterns of Gif contained 10 to 12 peptides in addition to those present in the Fc fragment (*10*). Since these findings suggested that Gif is an incomplete heavy chain, an attempt was made to identify the site and magnitude of the abnormality.

Because of a limited amount of material, we attempted to characterize all the cysteines of the molecule, particularly those in the hinge region. Amino acid composition of the oxidized protein revealed ten cysteic acid residues per heavy chain, based on an estimate of 360 residues per heavy chain. A peptic-tryptic diagonal map (*11*), obtained at *p*H 3.5, showed the presence of seven cysteic acid peptides. There were three pairs of linked cysteines plus one peptide which appeared unlinked. The three cystine bridges have been identified (see below) as the intrachain loop of the Fd variable region and the two intrachain loops of the Fc region (Fig. 1). The hinge peptide which contains four cysteines appears unlinked, since it is bridged to the identical peptide on the other heavy chain. We were unable to label any cysteines when the unreduced protein was alkylated in tris-guanidine, indicating the absence of any free SH groups.

Techniques for cleavage of the peptide chain by pepsin and trypsin and separation of the resulting peptides by high-voltage electrophoresis and radioautography have been described (*12*). Sequences of isolated radioactive peptides containing carboxymethylcysteine (CMCys) were determined by dansylation of amino terminal residues after sequential Edman degradation (Fig. 1). Four of the CMCys-containing peptides correspond to the two intrachain loops of the Fc fragment, while a fifth cysteine peptide has marked homology with the peptide that includes the cysteine at position 97 in the Fd variable loop (*13*). Peptide PT_1 with the composition Leu, Thr, $CMCys_{0.5}$ could include either the first variable cysteine at position 22 or the cysteine of position 367. Because of the size of the molecule and the presence of a third cystine bridge on diagonal maps we believe PT_1 and T_2 are linked and represent the intrachain disulfide loop of the Fd variable region. We were unable to identify the cysteine-containing peptides from the Fd constant region including the constant loop and the peptide joining the heavy to the light chain (Fig. 2B). This places the beginning of the deletion somewhere after residue 100, and probably at the beginning of the constant region of the Fd fragment. Since the sequence around the hinge region of a $\gamma 2$ myeloma protein (Sa) is known (*14*), we attempted to characterize this region of the molecule in the hope of characterizing the

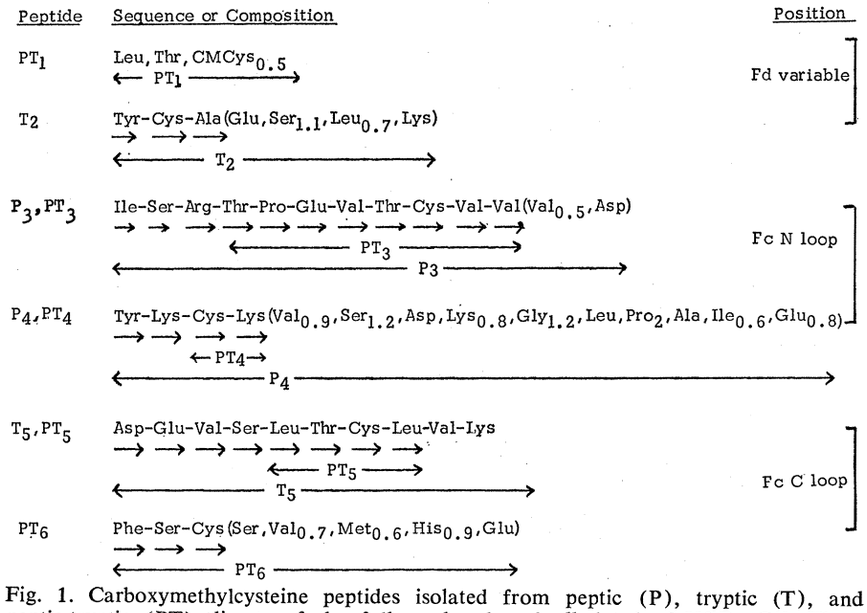

Fig. 1. Carboxymethylcysteine peptides isolated from peptic (P), tryptic (T), and peptic-tryptic (PT) digests of the fully reduced and alkylated $\gamma 2$ HCD, Gif. Three intrachain disulfide bridges of the molecule are present. No CMCys peptides corresponding to the Fd constant region were identified. The heavy-light peptide is absent, and the peptide containing the interchain bridges (linking the heavy chains) is shown in Fig. 2.

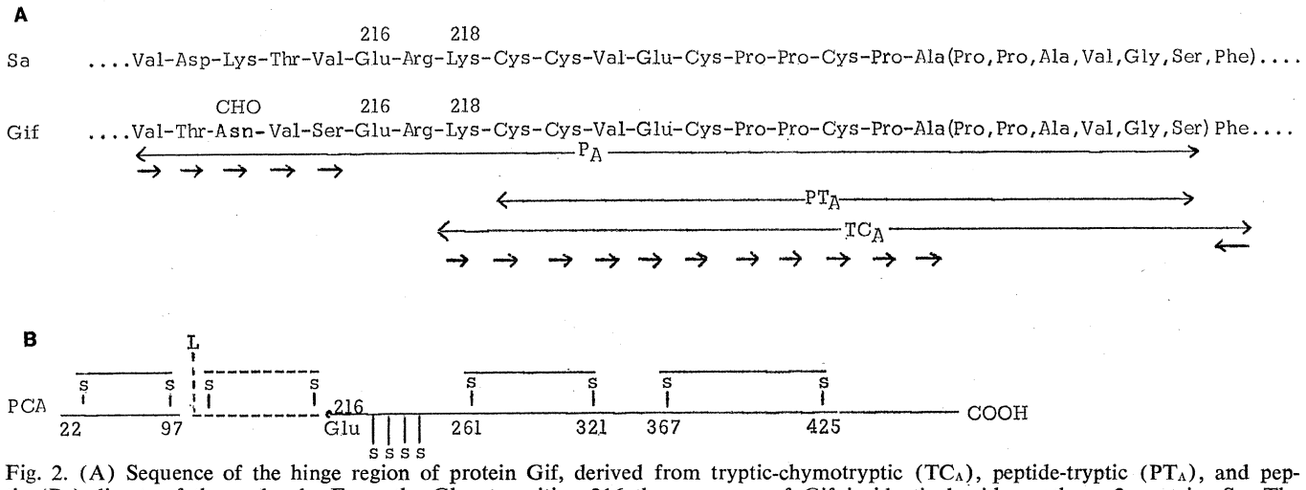

Fig. 2. (A) Sequence of the hinge region of protein Gif, derived from tryptic-chymotryptic (TC_A), peptide-tryptic (PT_A), and peptic (P_A) digests of the molecule. From the Glu at position 216 the sequence of Gif is identical with another $\gamma 2$ protein, Sa. The first five residues of P_A are not homologous with protein Sa, indicating that the normal sequence begins at position 216. Small arrows indicate residues sequenced in peptides P_A and TC_A. (B) Schematic representation of a $\gamma 2$ heavy chain monomer. The solid lines represent Gif and the dotted area is the deleted segment corresponding to the Fd constant region.

end of the deletion. A partial sequence of a peptide isolated from tryptic-chymotryptic and peptic-tryptic digests of the reduced molecule showed that the region containing the heavy chain bridges from 218 to 235 was identical to that found in Sa (*14*) (Fig. 2A). A related peptide (P_A), which contains several additional residues at the NH_2-terminal side, was then obtained (in low yield) from a peptic digest of the unreduced protein (*15*) by a series of steps that included high-voltage electrophoresis, descending chromatography with a butanol, acetic acid, water, pyridine system, and ion exchange chromatography (AG50W-X8, over a gradient of *p*H 3.1 to 3.7). Sequential Edman degradation revealed that the first five residues of P_A have the sequence Val-Thr-Asp-Val-Ser. These residues are not homologous with the region preceding residue 216 in protein Sa. Also, P_A contained carbohydrate, which must come from the amino terminus of the peptide since PT_A lacked carbohydrate (Fig. 2A). The composition of the remainder of this peptide from the Glu at position 216 to position 234 is identical with protein Sa. Not enough material was available to determine the sequence of Glu and Arg at residues 216 and 217. However, the location of the tryptic cleavage suggests that the sequence is as shown in Fig. 2A. A schematic representation of the molecule is given in Fig. 2B.

Gif differs from Zuc and Cra in the size of the deletion, but it resembles Hi in having a deletion of about 100 residues per heavy chain. As is shown by the sequences in Fig. 3, Gif is similar to Cra and Zuc in that normal sequence resumes at or around the Glu of position 216 and that carbohydrate is attached to Asn just before the regular sequence resumes. It would seem that more than coincidence must be involved in such a regular occurrence. If we suppose that the synthesis of the heavy chain is under the control of either a single or a series of closely linked genes, and that these arose by gene duplication from a smaller ancestral gene, then the sequence around residue 216 might be expected to be homologous with the NH_2-terminal of the heavy chain or the Fd constant region. However, this region which is known as the "hinge region" and contains the disulfide bridges linking the heavy chains is not homologous with any other region of the heavy or light chain and has been noted to contain the greatest subclass variability in IgG (*16*) and IgA (*17*). This lack of homology raises the possibility that it represents the product of a satellite DNA which has been inserted during the duplication of a primitive immunoglobulin gene. An alternative explanation for the observed lack of homology and great variability of this region is that, while it represents the results of gene duplication, it is unique because of still un-

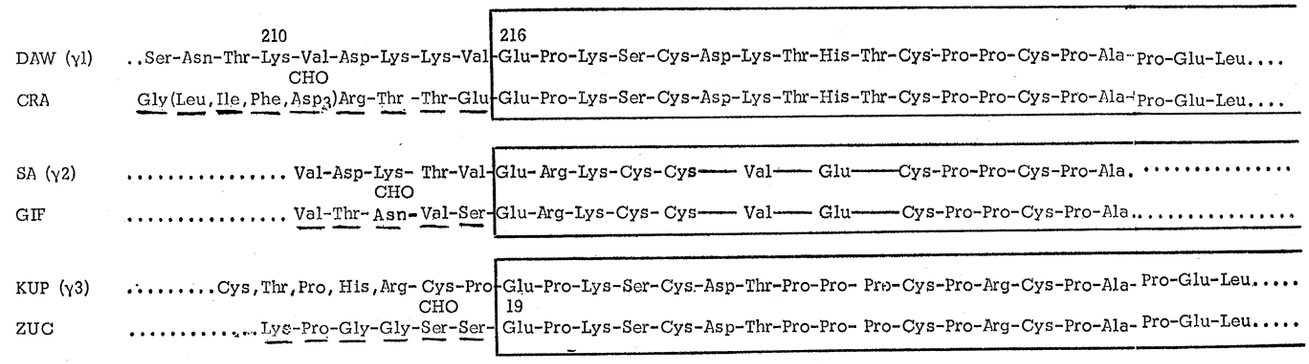

Fig. 3. The sequence from the hinge region of three HCD proteins as compared to the known sequence of proteins of the same subclass (*3, 13, 14*). All three HCD proteins begin regular sequence at position 216, and carbohydrate is attached to the molecule just before this site. Underscoring indicates the nonhomologous regions preceding the normal sequence.

known functional requirements that have permitted this degree of variability during evolution.

Since the four known subclasses of γ chains are under the control of a series of closely linked genes (*18*), it seems particularly striking that, as is shown in Fig. 3, γHCD proteins belonging to three of these subclasses have normal sequences after the same position. Thus it seems possible that Glu at residue 216 near the hinge region could represent the beginning of another gene. The data on two other immunoglobulins with large deletions do not provide further support for this possibility. The deletion in protein Hi has not been defined sufficiently to be informative (*7, 19*). Protein Sac, an unusual γG1 myeloma protein, has a light chain with an internal deletion of about 70 residues, and a heavy chain lacking the first 102 residues (*20*). The possibility that the findings in the heavy chain are the result of proteolysis has not been excluded. Smithies has proposed a branched-chain DNA for immunoglobulin genes, one that would result in varying deletions dependent on DNA breakage and differing mechanisms of repair (*21*). This could explain the results of the three HCD proteins if codon 216 was particularly susceptible to DNA breakage. The limited number of proteins studied makes any proposed mechanism highly speculative, but future studies may establish the reason for the unusual structure of the hinge region and provide the mechanism by which two, three, or more genes are integrated so that a single polypeptide chain is synthesized.

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4. Abbreviations are as follows: Ala, alanine; Asp, aspartic acid; Asn, asparagine; Arg, arginine; Cys, cysteine; Glu, glumatic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Pro, proline; Phe, phenylalanine; Ser, serine; Tyr, tyrosine; Thr, threonine; CHO, carbohydrate; PCA, pyrrolidinecarboxylic acid.
5. Numbering is based on the sequence of γ1 proteins because no γ2 myeloma has been completely sequenced. Protein Eu was described by G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, M. J. Waxdal [*Proc. Nat. Acad. Sci. U.S.* **63**, 78 (1969)]; protein DAW was described by Press and Hogg (*13*).
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15. Pepsin digestion was done in 0.02*M* HCl, *p*H 2, for 4 hours at 37°C, with addition of 0.1*M* HCl to keep the *p*H constant. After an additional 16 hours at 37°C, the digest was lyophilized and applied to a Sephadex G-50 column equilibrated with 5 percent formic acid. The material eluting as a small peak near the void volume was pooled and reduced and alkylated with [^{14}C]iodoacetic acid.
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22. We thank Dr. Donald Feinstein, University of Southern California, for making this protein available. Supported by PHS grants AM 2549, AM 1431, and AM 5064. B.F. is a senior investigator of the Arthritis Foundation, Inc.

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Acetylcholine Receptors: Number and Distribution at Neuromuscular Junctions in Rat Diaphragm

Abstract. *The number of acetylcholine receptors per motor end plate in the rat diaphragm, measured by the binding of [^{125}I]α-bungarotoxin, varies directly with rat size and is (4.0 ± 0.2) × 10^7 for full-grown male rats. Autoradiographic analysis of single fibers labeled with this substance reveals that virtually all of these receptors are localized in the end plate.*

α-Bungarotoxin (α-BGT), a polypeptide isolated from the venom of *Bungarus multicinctus*, binds tightly to skeletal muscle and electroplax membranes in the microregion of the acetylcholine receptors and blocks the response of these cells to acetylcholine (ACh) (*1–3*). The polypeptide binds specifically to ACh-sensitive cells (*4*), and both ACh and *d*-tubocurarine compete with α-BGT in the binding reaction (*1, 2*). For these reasons, α-BGT labeled with ^{125}I or ^{131}I is used as a tag for the ACh receptor. We have used [^{125}I]α-BGT to determine the number and spatial distribution of ACh receptors in the muscle membranes at rat neuromuscular junctions.

To determine the number of receptors per end plate, we pinned the left hemidiaphragms of male and female Sprague-Dawley rats (100 to 380 g) to stainless steel grids; in each hemidiaphragm, the endplate region of a layer of deep muscle fibers was exposed by dissecting along the phrenic nerve as it courses through the muscle mass. The pinned diaphragms were incubated for 2 hours at 37°C in modified Ham's F12 culture medium, which was buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid as zwitterion and contained [^{125}I]α-BGT, 10^{-6} g/ml. Then the diaphragms were transferred to fresh medium that lacked α-BGT, washed with 12 changes of medium for 5 minutes each, fixed in glutaraldehyde, and stained for the end-plate marker acetylcholinesterase (AChE) (*5*). Groups of muscle fiber segments containing 0 to 60 end plates were dissected from the muscle, the number of end plates in each group was determined, and each group was hydrolyzed in 100 μl of 6*N* HCl at 115°C for 48 hours. The hydrolyzates were dissolved and counted by scintillation spectrometry (*6*). Groups of muscle fibers that were free of end plates were analyzed to determine the correction factor for nonspecific binding (which was about 3 percent of total binding).

From 45 determinations on full-grown male rats (345 to 380 g), the number of ACh receptors ([^{125}I]α-BGT binding sites) per end plate was determined to be (3.98 ± 0.21) × 10^7 (mean ± standard error), and the range was (1.14 to 8.70) × 10^7 sites per end plate. Part of this variability is presumably due to variation in the size of individual end plates. The values determined for rats of various sizes are presented in Table 1. From the binding of [^{131}I]α-BGT to whole hemidiaphragms of rats (weight unspecified), Miledi and Potter (*2*) estimated a population of about 4.7 × 10^7 receptors per end plate.