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Determination of Primary Amino Acid Sequence and Unique Three-Dimensional Structure of WGH1, a Monoclonal Human IgM Antibody with Anti-PR3 Specificity[1]

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Transformed B cells making monoclonal IgM-λ anti-PR3 antibody WGH1 from a patient with Wegener's granulomatosis were used to prepare mRNA and synthesize cDNA. PCR primers for human μ and λ chains were then employed to amplify heavy- and light-chain V-regions followed by cloning into pCR2-1 vector and sequencing. Molecular modeling of VH regions employed knowledge-based homology modeling to obtain minimum energy conformation. The VH sequence was subgroup III with marked overall homology to VH1.9III. The VHCDR3 region of WGH1 was unique, consisting of 21 amino acid residues which included seven tyrosines as well as three negatively charged aspartic acid residues. The VL region was subgroup II with a negatively charged glutamic acid at position 100 in CDR3. Molecular modeling of VH revealed a major conformational difference in the shape of CDR3 compared with other antibodies for which three-dimensional structures have been determined. Monoclonal antibody WGH1 reacting with PR3 (a highly positively charged molecule) shows a unique reactive cassette within VHCDR3 with a number of negatively charged aspartic acid residues. WGH1 VHCDR3 contains a loop which shows a major projection not usually recorded in other previously studied antibody molecules.

INTRODUCTION

Wegener's granulomatosis (WG) is a systemic disease of unknown etiology characterized by necrotizing granulomas and vasculitis affecting the upper and lower respiratory tract and kidneys (1, 2). In most WG patients, presence of anti-neutrophil cytoplasmic antibodies (C-ANCA) provides a useful diagnostic serologic marker, often paralleling disease activity and tissue inflammatory reaction (3–5). Anti-neutrophil cytoplasmic antibodies react with a limited spectrum of neutrophil cytoplasmic antigenic materials, including proteinase-3 (PR-3) and in some instances myeloperoxidase or lactoferrin (6–9). Anti-PR-3 C-ANCA have been studied by a number of investigators, particularly for evidence as to whether they may participate directly in disease pathogenesis. Several possible mechanisms whereby antibodies with anti-PR-3 specificity might amplify or incite some of the tissue damage or inflammatory process in WG have been suggested (10–12), but there is very little immunohistochemical evidence for *in vivo* deposition of anti-PR-3 IgG or other antibodies reacting with neutrophil cytoplasmic components within pulmonary or renal lesions of WG or polyarteritis patients.

PR-3 remains one of the most prominent neutrophil cytoplasmic antigenic constituents reacting with anti-C-ANCA antibodies from patients with active Wegener's involvement. Previous studies have focussed on attempts to define the predominant antigenic epitopes on PR3 (13) or the structural features of V-regions of antibodies reacting with PR-3 or other related neutrophilic cytoplasmic antigens (14). In the present communication, we report the primary V-region sequence of a monoclonal human IgM anti-PR3 antibody derived from a cell line (WGH1) produced from a patient with WG and present molecular modeling constructs which show an unusual conformation within the heavy-chain V-region CDR3 of this human monoclonal antibody.

MATERIALS AND METHODS

Cell culture. Epstein–Barr virus–transformed B cells (WGH1) synthesizing IgMλ anti-PR-3 isolated from a patient with WG were prepared by E. Csernok in Lübeck, Germany, as previously described (15). The monoclonal IgM WGH1 showed strong ELISA reactiv-

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ity (OD_{490} 1.311) with PR-3, but negative ELISA reactions with both myeloperoxidose and lactoferrin. WGH1 mAb gave a strong positive C-ANCA pattern on immunofluorescence testing using human PMNS (Fig. 1). The cells were grown in RPMI 1640 medium with 10% fetal calf serum and L-glutamine. The adherent cells were collected by adding 0.02% EDTA to dislodge the cells and then counted (1.2×10^7 cells/ml). The cells were washed with phosphate-buffered saline, pelleted, and frozen at −70°C.

Isolation of mRNA. The isolation of mRNA from WGH1 was obtained through the use of the Fast Track mRNA Isolation Kit, Version 3.5 (Invitrogen, San Diego, CA). Frozen cells were lysed, and after washing DNA, proteins, and the nonpolyadenylated RNAs out with salt buffers, mRNA was eluted in the absence of salt. Eluted mRNA was precipitated with sodium acetate and ethanol, frozen, pelleted, and resuspended in 10 mM Tris, pH 7.5.

Synthesis of cDNA from mRNA. WGH1 cDNA was synthesized from mRNA by the use of the cDNA Synthesis Kit (Boehringer–Mannheim Biochemical, Chicago, IL). The first cDNA strand was synthesized by using oligo(dT) as a primer for AMV reverse transcriptase. Second-strand synthesis began with RNase H, which nicked the mRNA into fragments to be used as primers for the DNA polymerase I.

Polymerase chain reaction (PCR). Primers for the human mu and lambda chains were selected so that they would bind universally to the immunoglobulin variable regions in the forward and reverse directions. The primers used are shown below:

(A) Human mu (μ) chain (16)
   1. Forward primer: variable region amino acids Nos. 1–9
      5′-GGGAATTCAGGTGCAGCTG(GCT)-(AT)G-(CG)AGTC-3′
   2. Reverse primer: variable region amino acids Nos. 120–125 (17)
      5′-CC[AAGCTT]GAAGCTCCTCAGAGGA-GGG-3′

(B) Human lambda (λ) chain
   1. Forward primer, leader sequence (18)
      5′-GGGAATTCATG(AG)CCTG(CG)(AT)C(CT)CCTCTC(CT)T(CT)CT(CG)(AT)(CT)C-3′
   2. Reverse primer: variable region amino acids Nos. 120–125 (18)
      5′-CC[AAGCTT]GAAGCTCCTCAGAGGA-GGG-3′

Bases in parentheses represent substitutions at a given base and are present in equimolar amounts at that position. *Eco*RI sites are underlined and *Hind*III sites are in parentheses. An extra two Gs or Cs were added at 5′ of the restriction endonuclease sites to facilitate enzyme cleavage. The μ and λ regions were amplified using the Gene Amp PCR Core Reagents (Perkin–Elmer, Norfalk, CT) and the Perkin–Elmer Cetus DNA Thermal Cycler, with primers described above (16–18).

Cloning. All products were ligated into the pCR2.1 vector and then transformed into competent *Escherichia coli* cells using the Original TA Cloning Kit (Invitrogen, San Diego, CA). The plasmid DNA was extracted by a modified alkaline lysis procedure and then digested with *Eco*RI and *Hind*III. The digestions were screened by 2% agarose gel electrophoresis to ensure the presence of a 500-bp band.

Purification of ligated vector. Colonies containing the insert of interest were grown in 100 ml of LB + AMP medium to an $O.D._{600}$ 1–1.5 in preparation for Qiagen purification using the Qiagen Plasmid Maxi Kit (Qiagen Inc., Chatsworth, CA). The protocol was based on a modified alkaline lysis procedure, followed by the binding of plasmid DNA to a Qiagen anion-exchange column resin under well-defined salt and pH concentrations. Plasmid DNA was prepared for sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer) and the Perkin–Elmer Gene Amp 9600. Samples were then sequenced using an ABI 373A DNA sequencer. Sequencing was repeated three times for each Qiagen-purified product. The sequences were downloaded onto a Macintosh formatted disc, and the Sequence Editor (SeqEd) Program (Applied Biosystems, Foster City, CA) was utilized for editing, alignment, and visualization of the sequences. Kabat and Wu's "Sequence of Proteins of Immunological Interest" (19) then helped to classify the heavy and light chains. This entire process was repeated with a different selected colony to verify the sequence.

Homology modeling of variable region of heavy chain of WGH1. The sequence of the variable region of the heavy chain (VH1) from WGH1 was aligned using the homology module of Biosym (20) with sequences of VH1 regions of IgM molecule (1IGMH; PDB:1IGM) as a template from the Protein Data Bank (PDB) and other VH1 regions of IgG molecules for which crystal structures had been solved and their 3D structural information was available in the PDB. 1IGM was chosen as a template since the sequence of VH in WGH1 was most similar to 1IGM (21). From these alignment studies structurally conserved regions (SCRs) were identified. The coordinates of the SCR regions in VH1 of WGH1 were obtained from respective SCRs of 1IGM. In the case of nonidentical amino acid substitutions in SCRs, the coordinates of the backbone were taken from

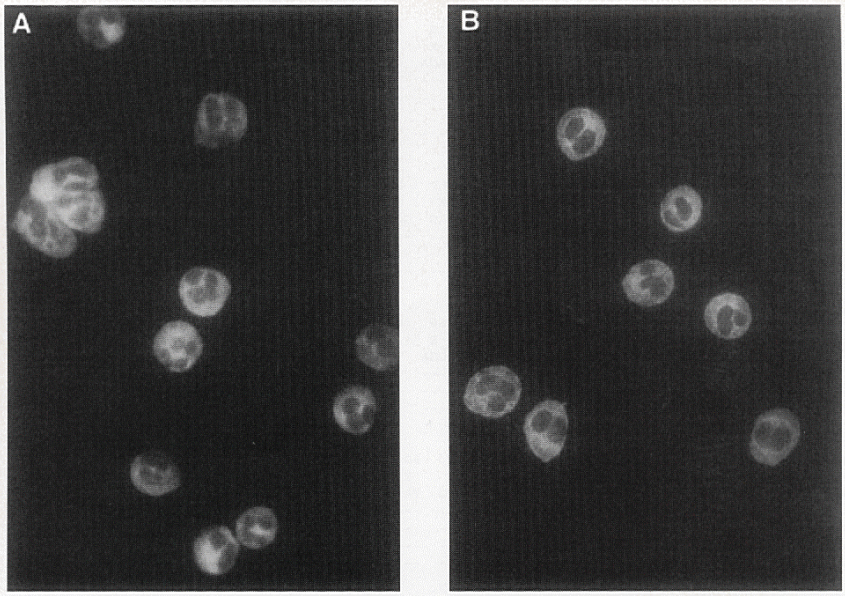


FIG. 1. (A) C-ANCA-positive staining by indirect immunofluorescence (IIF) of control human IgG anti-PR3. (B) C-ANCA-positive staining by IIF of IgM anti-PR3 from WGH-1.

the template protein and coordinates of the side-chains were assigned only after performing the conformational search procedure using the ROTAMER search library for the favored side-chain conformations (22, 23). Short contacts, if any, were removed by manually rotating side chains of the residues involved or by judicious choice of rotamers.

To assign initial conformation to loop regions, the database of known loop structures of proteins (24) was searched for loops of similar length. Five-residue-long regions on both sides of the loop were defined as flanking regions. Thus, for a loop between SCRn and SCRn + 1, the N-terminal flanking region would be the last five residues of the C-terminal portion of SCRn and the C-terminal flanking region of the same loop would be the first five residues of the N-terminal region of SCRn + 1. The rms deviation of the backbone atoms was calculated between the flanking regions of the loop from the database of loops and the flanking regions of the candidate loop. Only those loops with a rms deviation $\leq$2.0 Å were short listed. The conformation of the residues in the loops was further checked for the occupancy of (Φ, Ψ) angles in the allowed regions of the Ramachandran plot (25, 26). For example, to fix the conformation of the loop 97–109 in VH1 of WGH1, the search of "database of loops" gave 10 possible solutions using the above mentioned criteria of rms deviation $\leq$2.0 Å. The (Φ, Ψ) occupancy test gave the best solution for the initial conformation of the loop from the region 316–328 of hen egg ovalbumin (PDB:10VA(26)). Thus, main chain atoms of the loop 97–109 region were assigned the same conformation as that of residues 316–328 of hen egg ovalbumin. The conformation of the side chains in this loop was obtained by using the ROTAMER search criteria described above. The energy minimization was carried out for the loop region to improve and check the bond lengths, bond angles, and ω angles in the hinge regions of the candidate loop. The steepest descents and conjugate gradient minimization methods were used until the average rms derivative criteria reached 0.1 0.001 Kcal/mol/Å, respectively. The CVFF force field was used. No water molecules were explicitly taken into consideration, but to approximate the solvation effect, distance-dependent dielectric constant 4rij was used. The amino acid residues in the loop region were further subjected to molecular dynamics at 300 K for 500 ps to randomize the initial conformations of the residues in the loop region and to reduce the conformational bias. The dynamics were followed by minimization of the whole molecule using the steepest descents and conjugate gradient methods until the average rms derivative criteria reached 0.1 and 0.001 Kcal/mol/Å, respectively. In this way the steepest descent and conjugate gradient methods were used to carry out energy minimization and to obtain the most energetically stable conformation.

RESULTS

The complete nucleotide and the derived amino acid sequence of the VH region of WGH1 IgM anti-PR-3 is

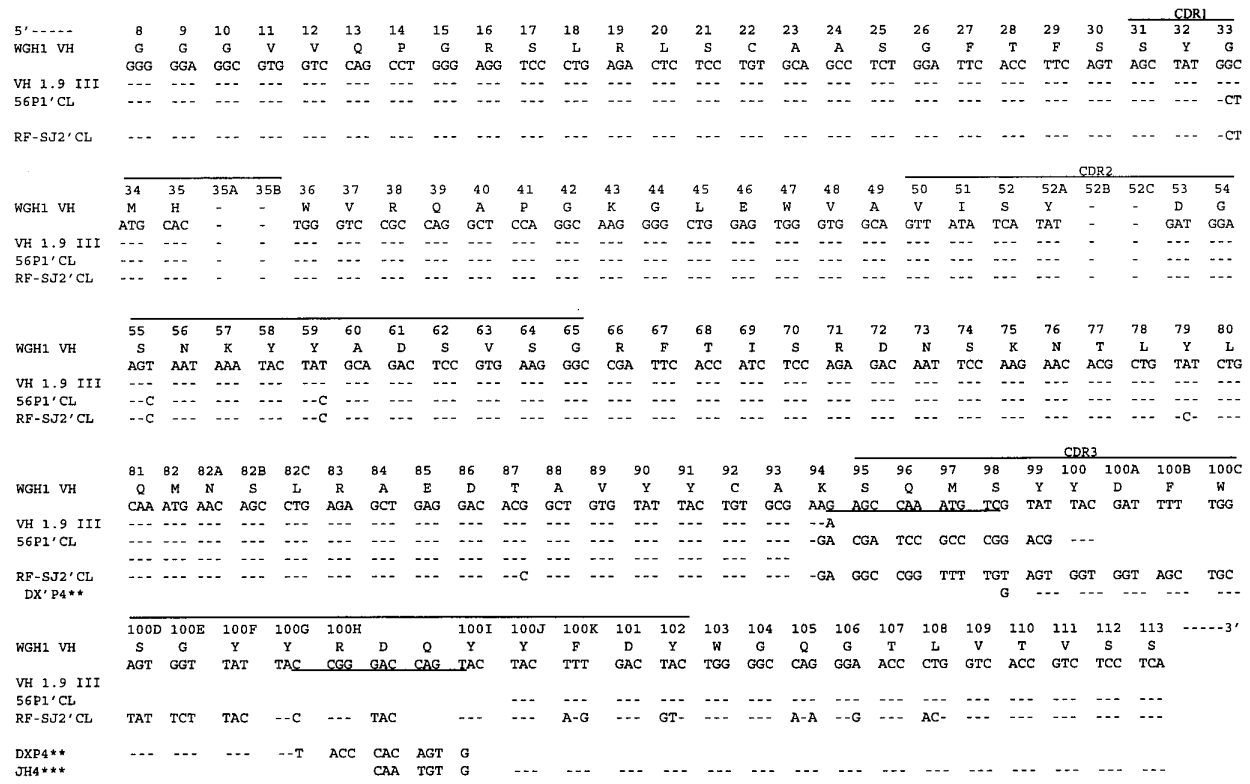


FIG. 2. Nucleotide sequence comparison of the WGH1 H chain (mμ). V segment to VH 1.9III, 56P1'CL, and RF-SJ2'CL. The deduced amino acid sequence for WGH1 is shown above the nucleotide sequence. Dashes indicate sequence identity with the residue with the line above. The CDR regions are indicated and possible N regions for WGH1 are underlined.

shown in Fig. 2. This sequence was VH 1.9III compared to the germline template in Kabat *et al.* (19) when amino acids 8–94 including FR1, CDR1, FR2, CDR2, and FR3 were examined. The WGH1 VH region showed 100% homology to the germline sequence of reference fetal antibody 56'CL within FR1, FR2, CDR2, and FR4 and 80% homology with CDR1 and 97% homology with FR3 of the reference fetal antibody. Comparison of the VH sequence of WGH1 to reference rheumatoid factor RF-SJ2'CL also showed 100% homology with FR1, FR2, and CDR2 with 80% homology between CDR1, 94% between FR3, and 82% between FR4. The VH region, therefore, was classified as subgroup III.

The VH CDR3 region of WGH1 appeared to be unique and consisted of 21 amino acid residues including seven tyrosines which accounted for one-third of the actual CDR as well as three interspersed negatively charged aspartic acids. A repeat motif of two adjacent tyrosines near a single aspartic acid was observed. The D minigene was DxP4 and the J minigene was JH4 or a JH5 variant.

The nucleotide and the derived amino acid sequence of the VL region of IGMλ anti-PR-3 WGH1 is shown in Fig. 3. The sequences were compared to DPL10 germline NEI Bence Jones protein in Kabat *et al.* (19). There was 89% homology between FR1, CDR1, FR2, CDR2, and FR3. The VL was classified as subgroup II. There was a 89% homology between the CDR regions. Notably, there was a glutamic acid at position 100 in the VLCDR3. The J minigene was JL2 or JL3.

MOLECULAR MODELING RESULTS

The Cα-trace of the VH1 of the WGH1 model is shown (white diagram) along with variable regions of heavy chains of the template IgM 1IgM (blue), IgM rheumatoid factor (orange), and other IgG molecules of known crystal structure (purple) in Fig. 4A. This model was evaluated in terms of steriochemical parameters

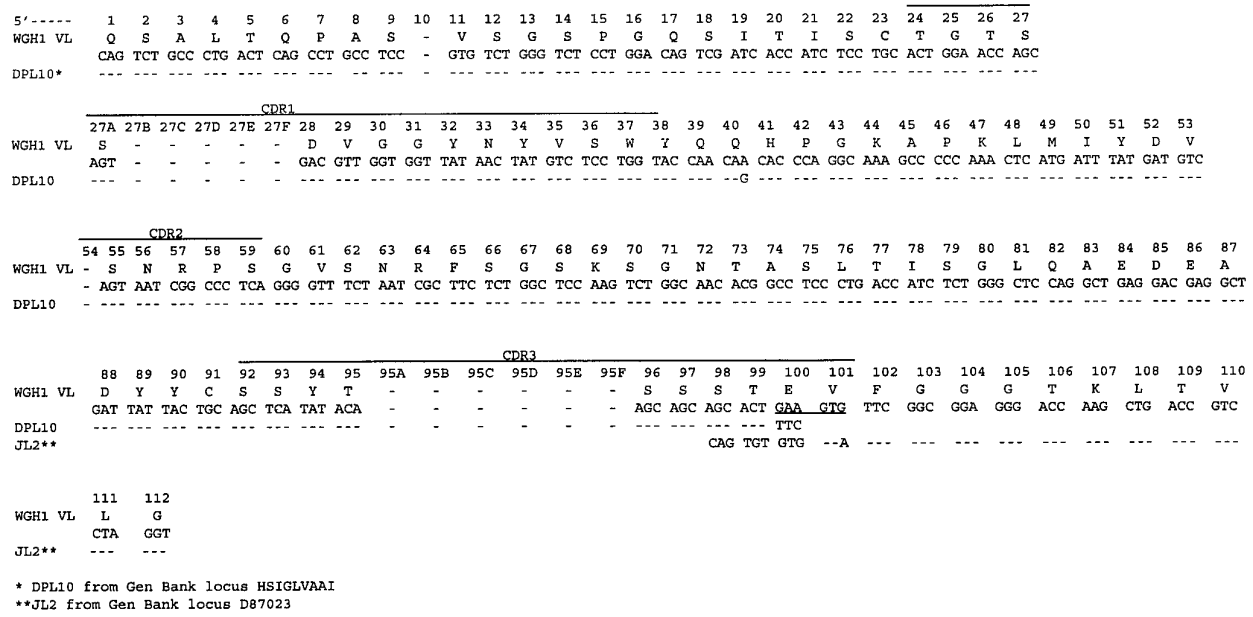


FIG. 3. Nucleotide sequence comparison of the WGH1 L chain (λ). V segment to DPL10. The deduced amino acid sequence for WGH1 is shown above the nucleotide sequence. Dashes indicate sequence identity with the residue with the line above. The CDR regions are indicated, and a possible N region for WGH1 is underlined.

such as bond lengths, bond angles, torsion angles, and packing environment and found to be correct from a steriochemical standpoint. The deviations were within the acceptable range. As can be seen from Fig. 5, (Φ, Ψ) values calculated for each amino acid residue of the model structure lay in the allowed region (extreme limit included) of the Ramachandran plot. The omega angles are close to 180° and no Van der Wall's overlaps were found. Evaluation of the 3D structure using a Cα-distance method developed inhouse (Kolaskar, Maithy, and Choudhary, unpublished data) gave a score of merit of 0.589, suggesting a good structure for the globular protein. The 3D structure was also evaluated using the WHAT IF method (27) and found to be a good model. The average packing environment score calculated using the WHAT IF method was −1.38 (which was very close to the observed packing environment score in well-resolved experimentally determined 3D structures). Thus, these evaluations point out that our model building studies provided a realistic structure of the VH region of WGH1. It can be seen from Figs. 4A and 4B that as in the case of the other immunoglobulin molecules, the major secondary structure was the β-sheet. However, there were distinct differences in the 3D structures of VH1 and variable regions of other IgM and IgG molecules. As can be seen in Figs. 4A and 4B, these differences were maximum within the CDR3 region. The conformation of the CDR3 region was not only different when compared to the CDR3 regions of other IgG and IgM molecules but also different compared to a model of the CDR3 region of a monoclonal rheumatoid factor recently studied by our group. Figure 4C shows a space-filling model of the CDR3 region DFWSGYYRDQYY which occupied a central position within the combining site loop on the surface of the VH region.

DISCUSSION

The sequence of the variable regions of the heavy chain as well as the light chain shown in Figs. 2 and 3 and its comparison with corresponding variable regions of immunoglobulin molecules point out that the major differences are in the CDR3 region of the heavy chain. Within the CDR3 region the hydroxy amino acids Ser and Tyr occur a large number of times. Similarly, the acidic amino acid Asp is also frequent. The presence of such a large number of hydroxy amino acids has given rise to a large loop structure of unique shape for a CDR3 region and the occurrence of a large number of negatively charged aspartic acid residues within the CDR3 may facilitate interaction with highly positively charged epitopes of the PR3 molecule.

It can be seen from Fig. 4C that the residues DFWSGYYRDQVY are involved in the formation of the loop which is on the surface of VH1 and thus is accessible for interaction with antigen. Accessibility of the CDR3 region is a major requirement in antigen–antibody complex formation since most models place the CDR3 in the center of the antibody combining site. The trace

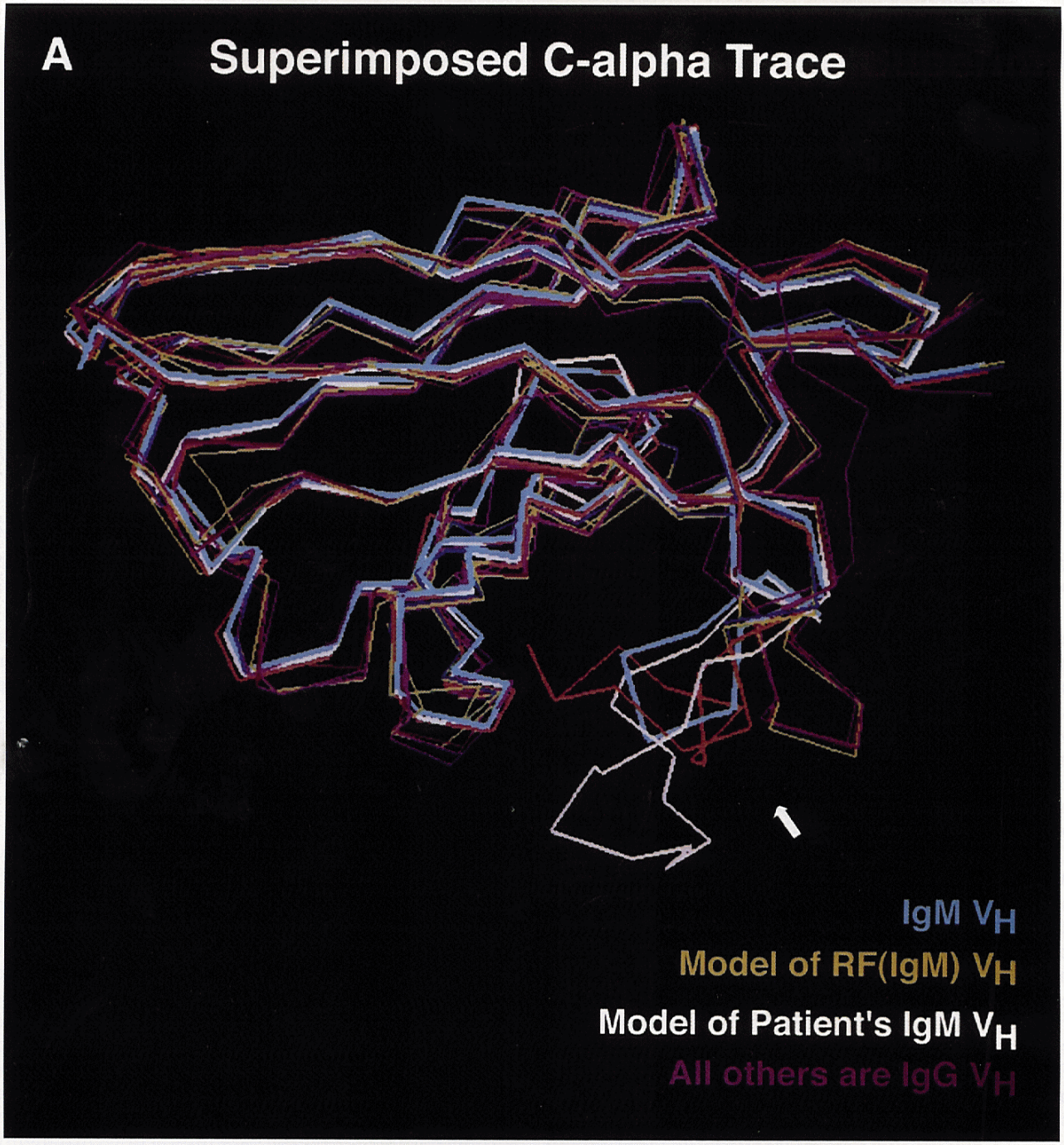
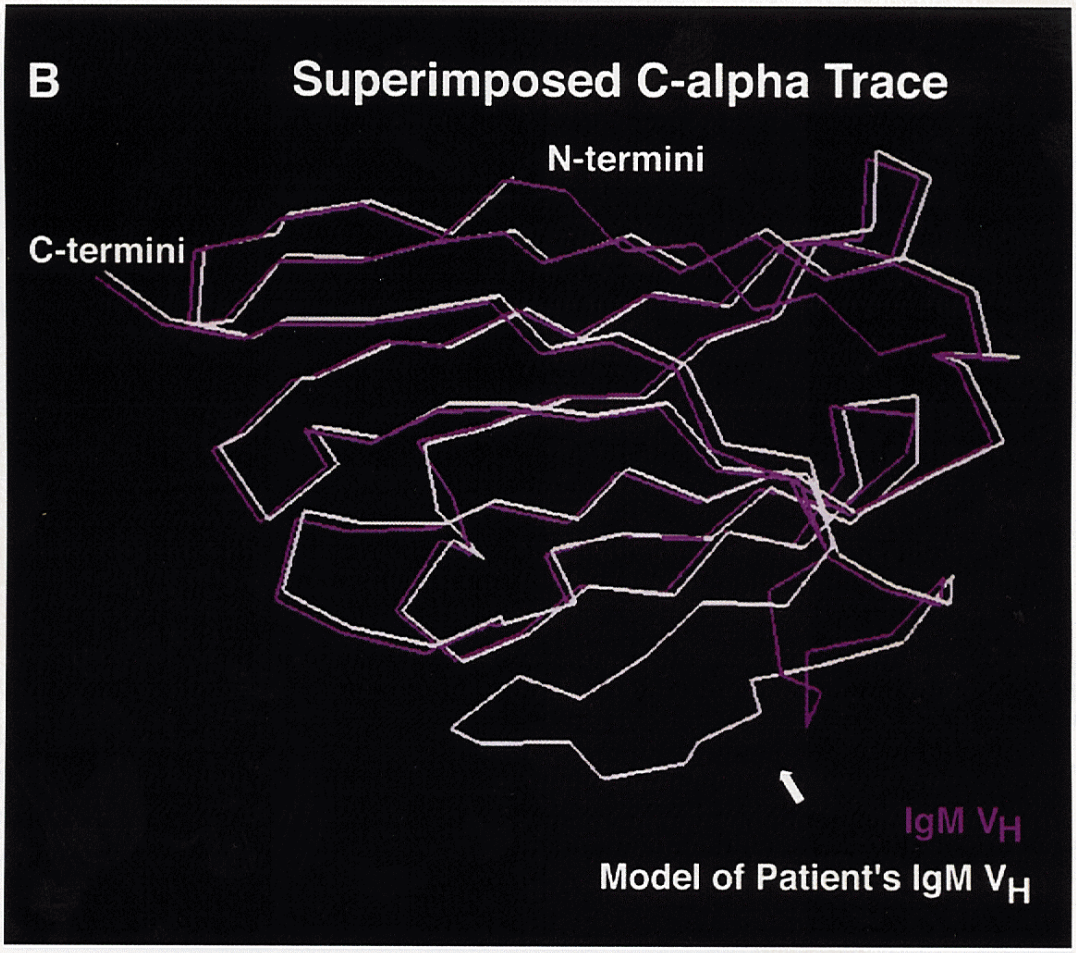


FIG. 4. (A) Superimposed C-α trace patterns of variable regions of heavy chains of IgM template molecule (PDB:1IGM) shown in blue, IgG (PDB:2GFB, 1DBB, 1DBA, 1IND, 1INE, 1DBK, 1, DBM) shown in purple, a model of a human rheumatoid IgM factor shown in orange, and WGH1 shown in white. Note the large loop size of WGH1 in the CDR3 region and the high similarity in 3D structure in other parts of the molecules compared. The view given is a frontal one with the VH CDR3 region being identified with a white arrow. (B) Comparative C-α trace of a Wegener's patient's VH WGH1 (white) compared with template IgM VH PDB:1IGM (purple) from the protein data base. Again the large white WGH1 CDR3 loop appears prominently at the lower part of the diagram. Again the CDR3 region is indicated by a white arrow. (C) Space-filling model of the variable heavy-chain region of monoclonal anti-PR3 WGH1. The color plate shown in the figure is used to represent the peptide (DFWSGYYRDQYY) forming CDR3 loops. It can be seen that these V-region heavy-chain residues are on the surface and are available for interaction with epitopes on PR3.



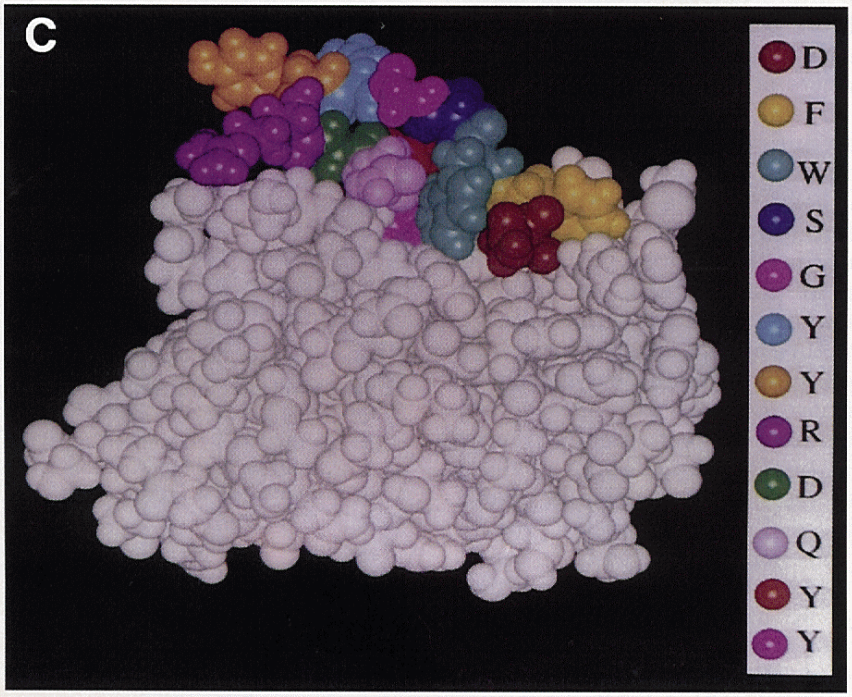


FIG. 4—*Continued*

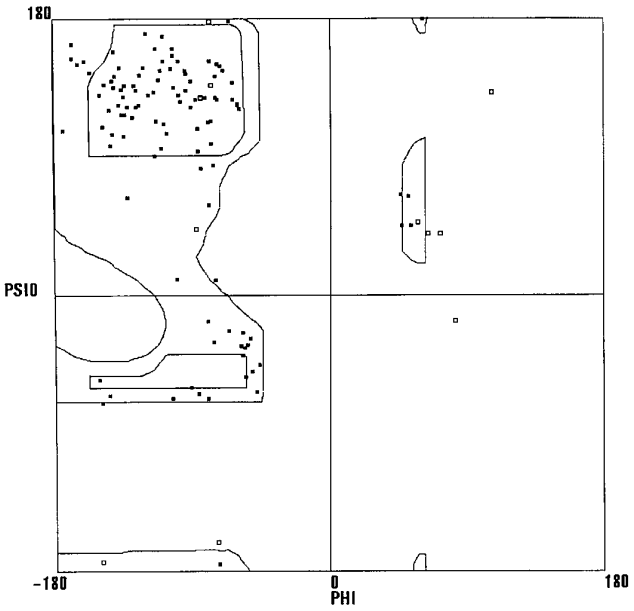


FIG. 5. Ramachandran plot of the WGH1 model using a program developed in-house. Note that all the non-Gly amino acids shown by filled squares are in the allowed regions. Gly residues are indicated as hollow squares and are also in the allowed region. Thus in the model every residue has taken an allowed conformation.

of the Cα atoms for various VH regions (see Fig. 4A) and the space-filling model shown in Fig. 4C point out the uniqueness of the conformation of the CDR3 region of WGH1. We suggest that this sequence (DFWSGYYRDQYY) may be interacting in complex formation with proteinase 3 (PR3). Support for this suggestion comes from the recently described crystal structure of RF-AN (28). The authors of this report (which is the first example of the crystal structure of an autoantibody reacting with an antigen) have pointed out that the antigen interacts most strongly with the CDR3 region of the autoantibody. In some other antigen antibody complexes often all six CDR regions may contribute almost equally in the complex formation. In WGH1, which is also an autoantibody, the CDR3 region is expected to be in major contact with its antigen PR3. The crystal structure of PR3, a serine proteinase within azurophilic granules of human polymorphonuclear neutrophils, is now available (29) and confirms many aspects of structure previously predicted in our modeling studies (13). In general, molecular modeling approaches have been reasonably accurate in predicting actual 3D structures of various globular proteins, but data concerning this point now need to be considerably extended. It is known from epitope peptide mapping studies that the PR3 antigenic determinants are situated mainly in the C′-terminal region (13). The loop region 108–124 of PR3 was shown to be most antigenic. Thus, in the complex of WGH1 and PR3 the residues from the CDR3 region and the amino acid residues 108–124 of PR3 will be interacting maximally. These interactions will probably be mostly in the form of hydrogen bonding and through the formation of one or two salt bridges. In any case, results presented here concerning the sequence of light and heavy chain variable regions of WGH1 and the modeling studies of the variable region of the heavy chain of WGH1 point out the unique sequence and 3D structure of the CDR3 region and now provide additional new data which may eventually help in understanding Wegener's granulomatosis and the role of PR3. Additional molecular modeling studies of the five IgM human monoclonal anti-PR3 antibodies recently described by Sibilia and co-workers (30) will be necessary before it can be ascertained whether the unique CDR3 structures of VH in WGH1 are also shared by other antibodies with this specificity.

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