Biochemistry

 Volume 17, Number 14 July 11, 1978

κ Chain Variable Region from M167, a Phosphorylcholine Binding Myeloma Protein†

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ABSTRACT: The amino acid sequence of the κ chain variable region from M167, a mouse myeloma protein, has been determined. M167 is a member of a group of seven murine phosphorylcholine binding proteins being studied in detail in an attempt to assess patterns of genetic diversity as well as structure–function relationships. To date this is the second light chain variable region from this group to be completed. A comparison with the previously determined M511 κ chain reveals that, although these proteins are members of the same "subgroup" or "variable region isotype" based on similar amino terminal sequences, a total of 11 amino acid interchanges were found (eight interchanges excluding acid–amide differences) of which only one occurred in a complementarity determining region. Of further interest has been the comparison of the M167 light chain with that of the human anti-phosphorylcholine myeloma protein FR. Considerable sequence homology was found between the frameworks and first complementarity regions of M167 and FR but little homology was observed in the second and third complementarity regions. The first complementarity regions of the phosphorylcholine binding proteins are the longest thus far determined in the mouse and yet M167 and FR have identical lengths in this area suggesting a strong selective pressure to maintain these structures in proteins with this binding specificity.

Antigen binding myeloma proteins derived from plasmacytomas induced in the BALB/c mouse have been studied for several years as models for the three-dimensional structure (Segal et al., 1974; Poljak et al., 1974; Padlan et al., 1976) and pattern(s) of diversity (Hood et al., 1970; Weigert et al., 1970; Hood et al., 1975; Rudikoff & Potter, 1976) of antibodies. One group of these proteins, those with specificity for phosphorylcholine, has been examined in considerable detail by several laboratories. Structural studies on the heavy chains from these molecules have shown that two (T15 and S107) are completely identical throughout the variable region (Rudikoff & Potter, 1976; Hood et al., 1975) while a third, H8 (Hood et al., 1975), differs at a single position in the third complementarity region. The heavy chain variable region from M603 (Rudikoff & Potter, 1974) while having an identical framework portion as T15, S107, and H8 has substitutions in the second complementarity region and substitutions as well as size differences in the third complementarity region. The same types of complementarity region differences are also seen in M167 (Rudikoff & Potter, 1976) and M511 (Appella, 1976) and in addition amino acid substitutions are found in the framework.

Based on partial amino-terminal sequences the light chains from the proteins with identical or nearly identical heavy chain sequences (T15, S107, and H8) show no differences through the first complementarity region (Barstad et al., 1974). In contrast, the κ chains from M603 and M167 are quite different in their amino-terminal portions from the T15, S107, H8 group and from each other. These two light chains have thus been assigned to different "subgroups" or "isotypes" (Hood et al., 1973). M167 and M511 differ at only a single framework position over their first 36 amino acids and are thus placed in the same subgroup. The similarity of M167 and M511 suggests that these two chains may be products of the same germ line gene. Thus, the comparison of these two sequences may provide insight into the mutational events occurring during somatic development.

Of further interest has been the comparison of the mouse anti-phosphorylcholine proteins with a human IgM Waldenstrom macroglobulin (FR) which also binds phosphorylcholine (Riesen et al., 1975). Riesen et al. (1976) found the N-terminal portion of the FR heavy chain to be remarkably similar to the same segment of the heavy chains from the mouse anti-phosphorylcholine proteins. Only four amino acid differences were found, three of which were located in the framework and one, an aspartic acid–glutamic acid interchange in the first complementarity region (H1). The complete light chain variable region of protein FR has been determined (Riesen & Jaton, 1976) and, while the framework portion is different from those of the mouse proteins, a striking similarity was found in both length and sequence of L1[1] when compared with M167.

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[1] L1, L2, and L3 refer to the three complementarity determining regions of the light chain. These areas form part of the surface of the antigen binding site (Segal et al., 1974) and exhibit the greatest degree of primary sequence variation (Kabat et al., 1976). The corresponding areas of the heavy chain which contribute the remainder of the antigen binding surface are designated H1, H2, and H3.

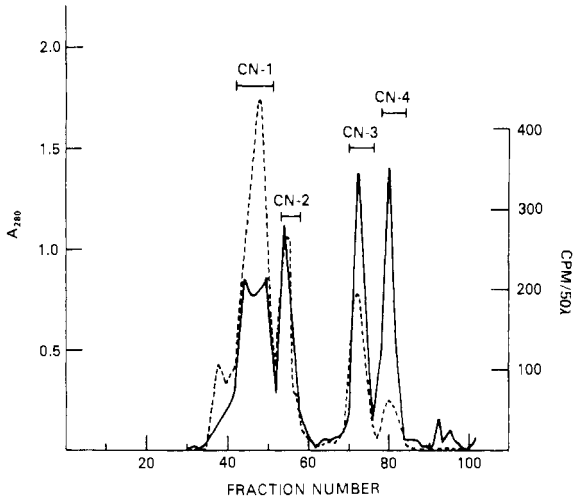

FIGURE 1: Sephadex G-100 gel filtration of fully reduced and ^{14}C alkylated light chains following CNBr cleavage. Column was equilibrated in 5 M guanidine-0.2 M NH_4HCO_3. A_{280} (- - -); cpm (—).

The complete heavy chain variable region from M167 has been previously reported (Rudikoff & Potter, 1976) and in the present communication we report the complete κ chain variable region sequence from M167. This sequence is compared with the light chains from the human FR and mouse 511 phosphorylcholine binding proteins.

Materials and Methods

Protein Purification. M167 was purified from ascites fluid on Sepharose-phosphorylcholine columns as described by Chesebro & Metzger (1972). Purified protein was dialyzed into 0.15 M Tris-HCl-0.15 M NaCl-2 mM Na_2EDTA and reduced with 10 mM dithiothreitol for 2 h at room temperature, followed by alkylation for 15 min with 20 mM iodoacetamide (Bridges & Little, 1971). The partially reduced and alkylated protein was dialyzed against 6 M urea-1 M HOAc and heavy and light chain separated on a Sephadex G-100 column equilibrated in the dialysis buffer.

Cyanogen Bromide Fragments. Light chains were dissolved in 70% formic acid and CNBr added at a 4:1 weight ratio (CNBr:protein). The reaction was allowed to proceed overnight at 4 °C after which the mixture was diluted with water and lyophilized. The lyophilized material was dissolved in 5 M guanidine-0.05 M Tris (pH 8.2), reduced with 10 mM dithiothreitol for 2 h at 37 °C, and alkylated with 20 mM [^{14}C]iodoacetamide. CNBr fragments were then separated on a Sephadex G-100 column equilibrated in 5 M guanidine-0.2 M NH_4HCO_3.

Succinylation and Tryptophan Cleavage. Light chains or CNBr fragments were dissolved in 5 M guanidine-0.05 M Tris-HCl adjusted to pH 9.0 and succinic anhydride added at a 100-fold excess over lysine residues. The pH was maintained at approximately 9.0 by the addition of 5 N NaOH and the reaction was allowed to proceed until the pH remained stable for 15 min. For tryptophan cleavage BNPS-Skatole (Pierce Chemical Co.) was used at 10 equiv per tryptophan essentially according to the method of Omenn et al. (1970). Protein was suspended in glacial acetic acid, BNPS-Skatole was added, and the suspension shaken to solubilize the reagent. The reaction mixture was then diluted to a final concentration of 80% acetic acid and shaken at 37 °C overnight. Following incubation an equal volume of water was added and the mixture extracted three times with chlorobutane. The upper layer containing excess reagent was discarded after each extraction and the sample then diluted with water and lyophilized.

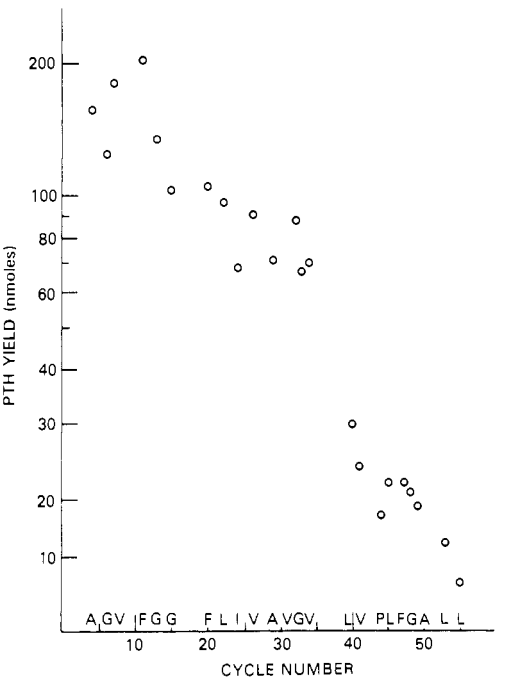

FIGURE 2: Semilog regression plot of selected amino acids calculated from gas chromatography analysis.

Sequence Determination. Automated degradations were performed on a Beckman 890C sequencer using a modified dimethylallylamine program as previously described (Vrana et al., 1977). The procedures used in this laboratory for identification of phenylthiohydantoin amino acids have been described in considerable detail (Rudikoff & Potter, 1976; Vrana et al., 1977) and consist of a combination of gas chromatography (Pisano et al., 1972), thin-layer chromatography (Summers et al., 1973), and amino acid analysis (Smithies et al., 1971). Certain residues were additionally subjected to high pressure liquid chromatography as described by Zimmerman et al. (1977).

Results

CNBr Fragments. Completely reduced and ^{14}C alkylated light chains were cleaved with CNBr and applied to a Sephadex G-100 column equilibrated in 5 M guanidine-0.2 M NH_4HCO_3. The major protein peaks were pooled as indicated in Figure 1, dialyzed against 0.2 M NH_4HCO_3, and lyophilized.

CN-1 was found by sequence analysis to be the N terminus of the light chain and from its size represents uncleaved light chains and possibly chains beginning at the N terminus and ending at the methionine at position 175 in the constant region. The amino-terminal 35 residues from this chain have previously been reported[2] with the exception of the Asx assignment at position 7 (Potter et al., 1976). Sequence analysis of CN-1 revealed position 7 to be Asp.

CN-2 was found to give a single sequence on preliminary analysis and was subsequently subjected to 56 cycles of degradation. A regression plot for a sequencer experiment on

[2] Positions 11 and 22 were originally reported as Asp (D) and Thr (T), respectively (Hood et al., 1973). We have since found these positions to be Asn (N) and Ser (S) (Potter et al., 1976). This reassignment has been independently corroborated (L. Hood, personal communication). Position 22 in M511 (Figure 3) has been reported as both Ser (Appella & Inman, 1973) and Thr (Appella et al., 1976).

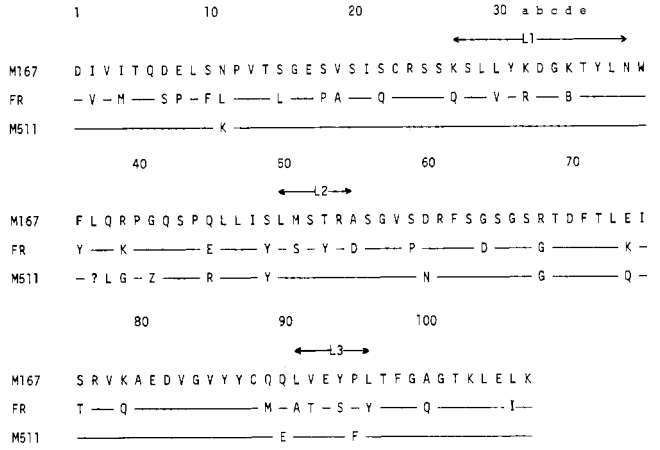

FIGURE 3: Comparison of the M167 κ chain with the light chains from the human protein FR and the mouse protein M511. Numbering is according to Kabat et al. (1976) in which the invariant tryptophan at the end of L1 is designated residue 35. M167 light chain has an insertion in L1 (designated 31 a–e) making it longer in this region than most κ chains.

CN-2 is given in Figure 2. This sequence can be placed by homology (Figure 3) as beginning in the middle of the second light chain complementarity region (position 52) and extending to the end of the variable region. The alignment of fragments in the sequence determination is given in Figure 4 and the actual sequences in Figure 3.

CN-3 was also a single fragment beginning by sequence analysis at the amino terminus. This fragment from its composition and size begins at the N terminus and ends at the methionine at position 51 in the second complementarity region (Figure 3). CN-4 by sequence analysis was a single peptide with an N-terminal sequence of Ser-Ser-Thr-Leu-Thr-Leu-Thr and begins at position 175 in the constant region.

Tryptophan Cleavage. In an attempt to complete the missing portion of the light chain (residues 36–51), an aliquot of CN-3 (positions 1–51) was succinylated, cleaved with BNPS-Skatole, and applied to the sequencer. In two experiments only the first four amino acids could be identified before the peptide apparently washed out of the cup. In a third experiment the BNPS-Skatole cleaved peptide was run in the presence of polybrene (Tarr et al., 1978) and all residues were identified to the C terminus of the peptide. The overlap between Trp-35 and Phe-36 was obtained by an amino-terminal sequencer run performed on native light chain. In order to obtain a fragment to provide an overlap between positions 51 and 52 succinylated light chains were cleaved at tryptophan residues with BNPS-Skatole and chromatographed on a Sephadex G-100 column in 5 M guanidine–0.2 M NH_4HCO_3. Peaks were pooled as indicated in Figure 5, dialyzed, and lyophilized. No sequence was obtained from BN-1 and this peak presumably represents succinylated, uncleaved light chain. BN-2 gave a single sequence beginning at Phe-36 and was continued to position 57, thus establishing the overlap between the NH_2 terminus and CN-2 (Figure 4) and confirming the sequence in this area. The remaining peaks (BN 3-5) were not examined further. From their size these pools are likely to contain fragments generated by cleavage at tryptophan residues in the constant region at positions 148 and 166 in addition to the amino-terminal fragment (1–35). Several other light chains containing only a single tryptophan in the variable region have been cleaved by this method and all give identical chromatographic profiles and sequence analysis (M. Vrana & S. Rudikoff, unpublished data). The yields from BNPS-Skatole cleavage appeared to range from approximately 30 to 50%.

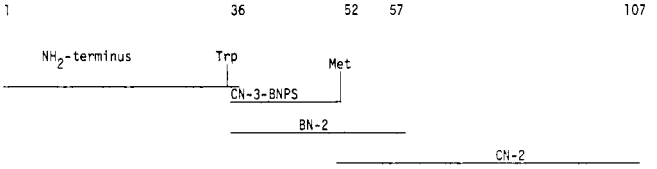

FIGURE 4: Schematic representation of the sequence determination of M167 κ chain variable region.

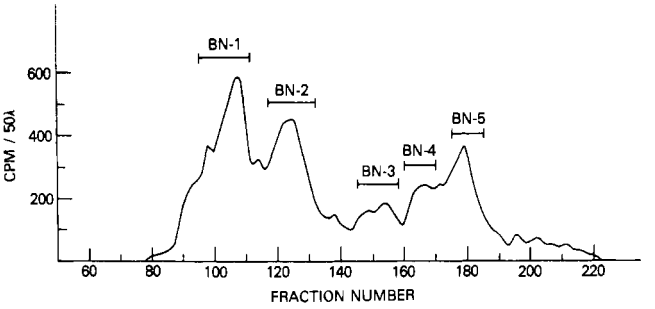

FIGURE 5: Sephadex G-100 gel filtration of succinylated, BNPS-Skatole cleaved light chain in 5 M guanidine–0.2 M NH_4HCO_3.

TABLE I: Homology (%) between M167 and Other Anti-Phosphorylcholine Light Chains.

	framework	L1	L2	L3
FR	75	69	33	33
M511	89	100	100	83
M603	64[a]	29	50[a]	
T15	46[b]	36		

[a] Comparisons with M603 from positions 36–90 are from S. Rudikoff, unpublished data. [b] Only positions 1–26 are available.

Discussion

The primary structure of the κ chain variable region from M167, a phosphorylcholine binding myeloma protein, has been determined by sequence analysis of the NH_2 terminus, a large CNBr fragment and a peptide produced by cleavage at tryptophan residues by BNPS-Skatole. The use of BNPS-Skatole to obtain sequence information after the constant Trp at position 35 may be of general use for light chains having only a single Trp in the variable region. Although cleavage in these experiments ranged from only about 30 to 50%, the ability to obtain an analyzable fragment with only a single gel filtration step (see Results and Figure 5) has considerable advantages over purification of smaller peptides. In fact, the peak used for sequence experiments most likely contains other peptides beginning at the NH_2 terminus and ending at Trp residues in the constant region. However, since the light chains were succinylated, these fragments are not available for Edman degradation and do not interfere with the sequence determination.

The comparison of the M167 light chain with that of the human anti-phosphorylcholine protein FR (Figure 3) reveals several interesting homologies. FR has been assigned to the human κ II subgroup by homology with other human light chains (Riesen & Jaton, 1976). M167 shows a 75% homology in the framework when compared with FR (Table I). The degree of framework homology between M167 and FR is considerably higher than between M167 and the V-κ regions from the other mouse anti-phosphorylcholine proteins M603 and T15. M167 thus may be the mouse analogue of human V-κ II subgroup. Anti-phosphorylcholine proteins in the mouse have

the longest first complementarity regions (L1) so far determined (Potter et al., 1976). It is striking that this region which evidences the greatest spectrum of size differences is identical in length in M167 and FR and displays a 69% sequence homology, whereas the homology with proteins M603 and T15 is 29 and 36%, respectively. The long L1 has been found in the three-dimensional structure of M603 to interact with the third heavy chain complementarity region in the formation of the combining site and may be essential in all phosphorylcholine binding proteins (Segal et al., 1974; Padlan et al., 1976). Thus, this structure may be under strong selective pressure resulting in the remarkable conservation seen between these two evolutionary distant species. In contrast, L2 and L3 show very little homology between M167 and FR (Table I).

McKean et al. (1973) have previously compared κ chains from four proteins with identical amino-terminal sequences to Cys_{23} in an assessment of variation within a "subgroup" or "isotype". Two of the chains differed by three residues, the third by eight and the fourth by 20–21 suggesting the presence of at least two germ line genes coding for these proteins. It should be noted that these light chains were all Bence-Jones proteins selected on the basis of their amino-terminal sequences and had no known antigen binding specificity. A comparison of M167 with M511 again selects two proteins from the same "subgroup" but additionally adds the constraint that these proteins both bind phosphorylcholine. When these two light chains are compared, 11 amino acid differences are observed (Figure 3), only one of which is in a complementarity region. Three of the interchanges result from differences in acidic and amide residues in the two proteins (positions 60, 74, and 90). Assignment of acidic vs. amide residues can be quite difficult as considerable deamidation may occur during sample work-up. Thus, the possibility exists that the differences at these positions result from technical problems in identification. This difficulty is important in that we have previously demonstrated that the M167 and M511 light chains have identical mobilities by isoelectric focusing (Rudikoff & Claflin, 1976). From the sequences it can be seen that M167 has one more negative charge in the variable region than M511. Therefore, assuming the constant regions are identical in charge either isoelectric focusing cannot distinguish between a single charge difference or there are mistakes in charge assignments in one or both proteins which as discussed above are difficult assignments to make. If the acids–amides at positions 60, 74, and 90 in the two proteins were in fact identical, then the amino acid differences involving charged residues in M167 (Arg-39 and Arg-68) would be compensated by comparable changes in M511 (Lys-11 and Arg-45) resulting in overall identical charges in the two proteins. For the M167 chain all positions at which sequence differences occurred when compared with M511 were analyzed in two separate preparations by both thin-layer and high pressure liquid chromatography. It should be noted that the M511 sequence appears only in summary form (Appella et al., 1976) which makes these comparisons somewhat difficult.

Of the 11 amino acid differences found between the two chains only the Pro-Phe interchange at position 95 in L3 would require two nucleotide changes to account for the observed amino acids. All other substitutions can be accounted for by single nucleotide changes and occur in the framework. If one excludes the three acid–amide differences discussed above, a total of nine mutational events would be necessary to derive one of these light chains from the other. This number is similar to that observed by McKean et al. (1973) in the comparison of M321 and T124 with M63. Thus, if these proteins were to be considered somatic derivatives of the same germ line gene it seems that the number of mutational events possibly occurring in the ontogeny of this lymphocyte line must be at least of the order compatible with the differences observed above. It should also be noted that in distinction from mouse λ chains where essentially all substitutions occur in complementarity regions (Weigert et al., 1970) all but one of the differences in the M167–M511 proteins occur in the framework. It therefore seems unlikely that mutation in this system would be selected for by antigen. Similarly, we have found in a study of the heavy chain variable regions from four levan binding myeloma proteins that the six substitutions observed all occur in the framework (Vrana et al., 1978). An alternative interpretation would be that M167 and M511 are coded for by separate germ line genes. If this were the case, and in addition the proteins analyzed by McKean et al. (1973) required at least two germ line genes, then based on these limited examples it would appear that in the mouse κ system the number of germ line genes would be extremely large.

Acknowledgment

We thank Ms. Mary Heller for excellent technical assistance during the course of these experiments and Ms. Linda Brunson for help with the preparation of this manuscript.

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Location and Interactions of Phospholipid and Cholesterol in Human Low Density Lipoprotein from ^{31}P Nuclear Magnetic Resonance†

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ABSTRACT: The major phospholipids, phosphatidylcholine and sphingomyelin, of low density lipoprotein (LDL) are accessible to small amounts of Pr^{3+}, suggesting that the head groups of all mobile phospholipids are on the surface of the particle in contact with the aqueous medium. The major source of the nuclear Overhauser effect enhancement of ^{31}P resonances is the *N*-methyl proton of the choline moiety, indicating close *N*-methyl phosphate group interactions, probably similar to those found previously in phospholipid vesicles. This behavior of the phospholipid head groups in LDL is similar to that in small vesicles without cholesterol, suggesting that in LDL most of the cholesterol is not associated with mobile, surface phospholipids. In contrast to LDL, where the presence of a large protein immobilizes some phospholipid head groups, immobilization does not occur in high density lipoprotein, consistent with occurrence of smaller peptides in the latter.

Human low density lipoprotein (LDL)[1] is the major serum carrier of cholesterol, plays a role in the control of biosynthesis of cholesterol, and is implicated in the formation of atherosclerotic plaques. The overall molecular organization suggested is that of a spherical particle with a neutral lipid core surrounded by phospholipids and protein with their polar portions encountering the solvent (for a recent review, see Morrisett et al., 1975). Such molecular details have only begun to be established experimentally, however. The most recent small-angle x-ray scattering data have been interpreted as arising from a monolayer of phospholipid and protein around a core of cholesterol esters containing some triglyceride (Deckelbaum et al., 1975; Tardieu et al., 1976; Deckelbaum et al., 1977). The cholesterol esters in the core are sufficiently organized to undergo a phase transition (Atkinson et al., 1977).

In order to obtain more molecular information, we have explored the behavior of the phospholipid portion of LDL using ^{31}P NMR. Since the only significant phosphorus in the particle is in the phospholipid, no ambiguities exist concerning the location of this nonperturbing probe. Previously it was found that about one-fifth of the phospholipid of LDL is immobilized by the B protein (Yeagle et al., 1977a). In the present study, the evidence suggests that all the mobile phospholipid head groups reside on the surface of the particle, that the unesterified cholesterol may not be associated with the surface phospholipids, and that the head-group conformations of the major phospholipids are similar to their conformations in phospholipid vesicles.

Materials and Methods

Human plasma low density lipoprotein ($1.019 < d < 1.063$) was isolated from several donors and characterized as previously described (Yeagle et al., 1977a). Human high density lipoprotein ($1.063 < d < 1.21$) was isolated by flotation, washed, and further purified on a Sepharose CL-6B column. Their purity was evaluated by immunoelectrophoresis and by $NaDodSO_4$-polyacrylamide gel electrophoresis as previously described (Yeagle et al., 1977a). Egg phosphatidylcholine and brain sphingomyelin were purchased from Avanti Biochemicals and cholesterol from General Biochemical. TRTPCK

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[1] Abbreviations used are: LDL, low density lipoprotein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOEE, nuclear Overhauser effect enhancement; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate.

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