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κ Chain Variable Regions from Three Galactan Binding Myeloma Proteins†

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ABSTRACT: A series of seven BALB/c myeloma proteins has been identified with binding specificity for antigens containing β(1 → 6)-D-galactopyranosyl moieties. We have determined the primary amino acid sequence of the first 108 residues from the light chains of three of these proteins. The framework portions of the variable regions of these three light chains are identical with residue 100 at which position three different amino acids are found in the three chains. An additional interchange was found at position 106 in one of the proteins. Based on recent DNA sequence studies suggesting that the variable region ends at residue 97, these substitutions indicate the possible existence of multiple genes coding for the region beginning at residue 98 and continuing toward the carboxy terminus. A single amino acid interchange was observed in complementarity determining regions occurring in L3. This substitution (Ile-Trp) would require changes in all three codon bases to produce the respective amino acids if one were derived from the other. Two of these chains are thus indistinguishable for their first 100 amino acids and are the first pair of κ chains to exhibit complete identity over their variable regions.

One of the oldest and most intriguing problems in immunology is the question of the mechanism(s) involved in the generation of the vast array of antibody diversity. For the past several years attempts have been made to approach this problem by amino acid sequence analysis (Hood et al., 1970, 1976; Weigert et al., 1970; Rudikoff & Potter, 1976; Haber et al., 1976; Capra et al., 1976; Weigert & Riblet, 1976) and nucleic acid hybridization studies (Leder et al., 1976; Tonegawa et al., 1976). The availability of inbred mouse strains and the ability to induce myeloma proteins in certain of these strains (Potter, 1972) has greatly facilitated these experimental approaches. The use of this system has thus permitted an evaluation of protein structures derived from a genetically homogeneous population and avoids many of the problems introduced by genetic polymorphism in outbred populations.

The initial studies on mouse λl chains by Weigert et al. (1970) demonstrated that, in the case of λl where a total of 18 proteins have now been sequenced, 11 are identical throughout the variable region. The remaining seven differ by no more than three amino acids with all substitutions occurring in complementarity determining regions (CDR). This result led these investigators to suggest that a single germ line gene coded for λl chains and that the variants arose by somatic mutations which could then be clonally expanded by antigenic selection. This conclusion was extended to propose that the number of germ line genes was small and that diversity was generated through somatic mutation.

Results from studies on mouse κ chains reveal a much more complex system as evidenced by the large number of mouse κ isotypes or subgroups, each of which presumably requires a

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minimum of one germ line gene. From published data 28 different V_κ isotypes or subgroups have been found among 65 sequences (Hood et al., 1970; Appella & Inman, 1973; Potter et al., 1976a). These isotypes were determined from a relatively nonrandom set and are thus likely to present a minimal estimate of the total number of V_κ isotypes coded by the BALB/c genome. Further evidence also suggests that multiple germ line genes may be required to code for a given V_κ isotype (McKean et al., 1978). Whether this phenomenon will hold true for all V_κ isotypes remains to be seen.

To date, relatively few sequences are available from κ chains derived from proteins with known antigen binding specificity. This laboratory has for some time been involved in an analysis of groups of proteins with antigen binding activity to chemically defined antigenic determinants including phosphorylcholine, levan, and galactan (Potter et al., 1976b, 1977). We have previously reported the comparison of two κ chains from phosphorylcholine binding proteins which are members of the same isotype group (Rudikoff & Potter, 1978). In the present communication we describe structural studies on the light chains from three myeloma proteins (X-24, X-44, and T-601) with $\beta(1 \rightarrow 6)$-D-galactopyranosyl binding specificity. A total of seven BALB/c myeloma proteins with this specificity have now been identified and partially characterized (Rudikoff et al., 1973; Jolley et al., 1973, 1974; Manjula et al., 1976; Mushinski & Potter, 1977). Previous sequence analysis has shown that κ chains from all $\beta(1 \rightarrow 6)$-D-galactan binding myeloma proteins are members of the same isotype group and, in fact, have amino terminal sequences identical with Cys_{23} (Rudikoff et al., 1973). We now report complete variable region sequences from three of the galactan binding myeloma proteins and compare the pattern of variation observed with other mouse light chains.

Materials and Methods

Protein Purification. $\beta(1 \rightarrow 6)$-D-Galactan binding myeloma proteins T-601, X-24, and X-44 were purified from ascites fluid on Sepharose–bovine serum albumin–*p*-azophenyl β-D-thiogalactopyranoside columns according to methods previously described (Potter & Glaudemans, 1972).

Light Chain Preparation. Purified proteins (25–35 mg/mL) were dialyzed against 0.15 M Tris-HCl–0.15 M NaCl–2 mM Na_2EDTA, pH 8.0, and partially reduced with 10 mM dithiothreitol for 2 h at room temperature in the dark, followed by alkylation with 20 mM iodoacetamide for 15 min. Partially reduced and alkylated proteins were dialyzed overnight against 6 M urea–1 M HAc and heavy and light chains separated on a Sephadex G-100 column equilibrated in the dialysis buffer.

Cyanogen Bromide Cleavage. Light chains were dissolved in 70% formic acid and CNBr was added at a 4:1 weight ratio (CNBr:protein). The reaction mixture was allowed to stand overnight at 4 °C, then diluted with water, and lyophilized. The CNBr cleaved material was dissolved in 5 M guanidine–0.2 M NH_4HCO_3 and fragments were separated on a Sephadex G-100 column equilibrated in the same buffer.

Succinylation and Trypsin Cleavage. Appropriate CNBr fragments were dissolved in 5 M guanidine–0.05 M Tris-HCl adjusted to pH 9.0. Succinic anhydride was added at a 100-fold excess over free NH_2 groups and the pH maintained at approximately 9.0 for 6 h by the addition of 1 M NaOH. The succinylated peptide was dialyzed against 0.2 M NH_4HCO_3 and lyophilized. This material was then dissolved in 0.1 M NH_4HCO_3, digested with trypsin (1:50) for 2 h at 37 °C, and lyophilized.

Sequence Determination. Automated degradations were performed on a Beckman 890C sequencer using a modified dimethylallylamine program as described by Vrana et al. (1977). The conversion of sequencer derivatives to phenylthiohydantoin-amino acids and their subsequent identifications have been described in considerable detail (Rudikoff & Potter, 1974, 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 of the HI hydrolysis products of the phenylthiohydantoin-amino acid (Smithies et al., 1971). More recently we have begun to use high-pressure liquid chromatography (Zimmerman et al., 1977) as an alternative to gas and thin-layer chromatography. All positions in the latter portion of any sequencer run are hydrolyzed and subjected to amino acid analysis in addition to assay by the various other techniques.

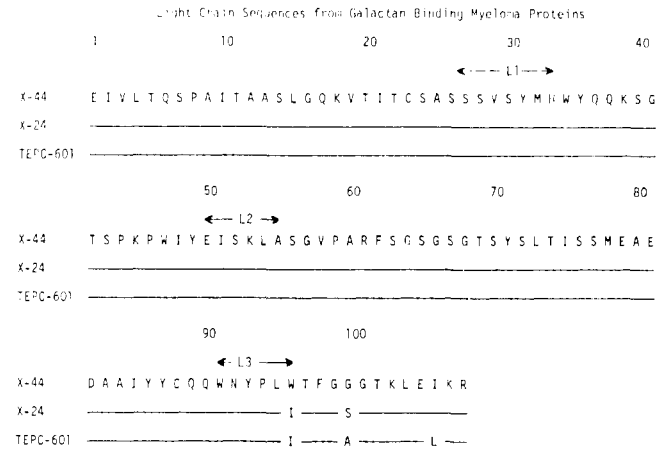

FIGURE 1: Comparison of the amino acid sequence of the κ chains from the three galactan binding myeloma proteins. Numbering is according to Kabat et al. (1976) with the invariant Trp at the end of L1 designated as position 35. The anti-galactan light chains thus have a one residue deletion in L1 in this numbering scheme when compared with other light chains.

Results

NH_2-Terminal Sequences. Intact light chains from the three proteins were subjected to 34–36 cycles of degradation and the sequences obtained are given in Figure 1. The identification of a methionine residue at position 32 in each chain suggested the use of CNBr to obtain additional fragments. The succeeding results were identical for all three proteins and will therefore only be described for T-601.

CNBr Fragments. Partially reduced and 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 elution profile is given in Figure 2. The four peaks obtained were pooled as indicated, dialyzed against 0.2 M NH_4HCO_3, and lyophilized. Sequence analysis of F1, F2, and F3 indicated the presence of three peptides consisting of fragments representing positions 1–32, 79–175, and 176–214 in each peak. These fragments would be expected to still be linked by disulfide bonds and thus chromatograph in the same peaks as observed. The difference in size between F1, F2, and F3 is presumably due to aggregation.

Sequence analysis of F4 revealed a single peptide beginning at position 33 and by composition consisting of 45 amino acids; 400–480 nM of this fragment (residues 33–78) was applied to the sequencer and subjected to 42 successful cycles of degradation. A regression plot for a degradation of F4 is presented in Figure 3 giving quantitative data on a typical sequencer experiment. The repetitive yields from two runs on this peptide

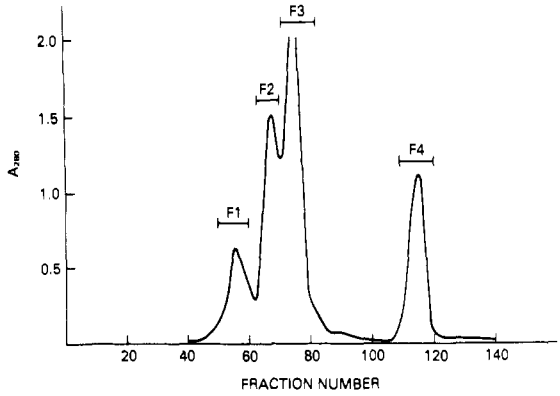

FIGURE 2: Sephadex G-100 gel filtration in 5 M Gn–0.2 M NH_4HCO_3 of anti-galactan light chains following CNBr cleavage.

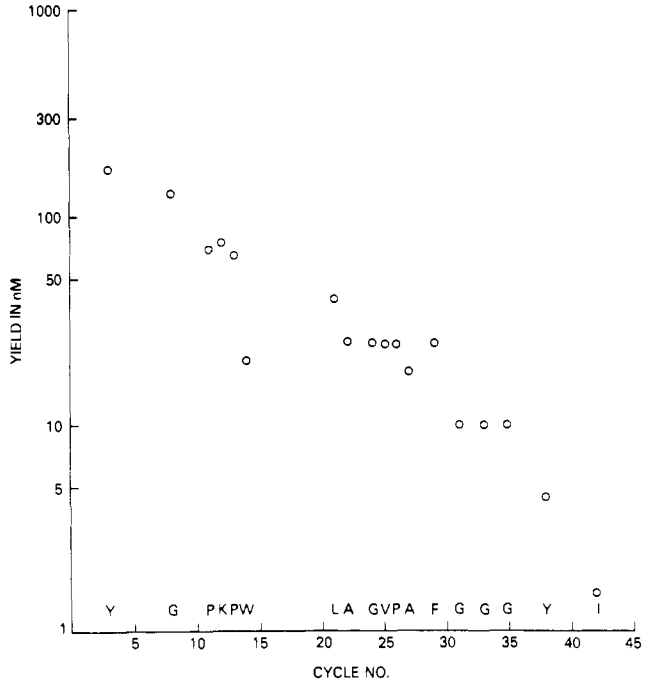

FIGURE 3: Semilog regression plot of a representative sequencer experiment on CNBr fragment F4. Data were calculated from high-pressure liquid chromatograph analysis.

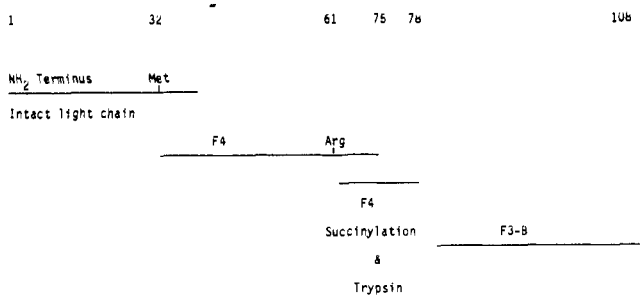

FIGURE 4: Schematic representation of the sequence determination of anti-galactan light chains.

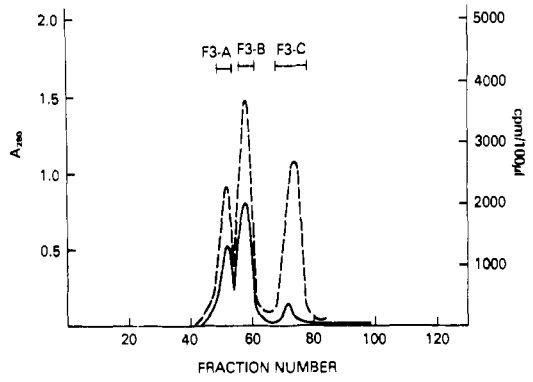

FIGURE 5: Sephadex G-100 gel filtration of CNBr fragment F3 following complete reduction and ^{14}C alkylation. Column was equilibrated in 5 M Gn–0.2 M NH_4HCO_3; A_{280} (—); cpm (- - -).

were 92.5 and 92.9%. The sequence obtained from this fragment is given in Figure 1 and schematically represented in the sequence determination in Figure 4. The carboxy-terminal 3 residues of this peptide were determined by the following procedure. An aliquot of F4 was succinylated to block both the NH_2 terminus of the peptide and free ε-amino groups of lysine. The peptide was then digested with trypsin resulting in enzymatic cleavage at the arginine at position 61. The digest was applied to the sequencer in the presence of 3–4 mg of polybrene (Tarr et al., 1978) and the sequence determined from position 62 to the C-terminus, position 78 (Figures 1 and 4).

The fragment beginning at residue 79 and containing the remainder of the variable region had previously been identified in peaks F1, F2, and F3 following gel filtration of CNBr cleaved light chain (Figure 2). Since F3 contained the largest amount of material, this peak was dissolved in 5 M guanidine–0.05 M Tris (pH 8.2), fully reduced with 10 mM dithiothreitol for 2 h at 37 °C and alkylated with 20 mM [^{14}C]iodoacetamide for 30 min. F3 was then reapplied to the Sephadex G-100 column in 5 M guanidine–0.2 M NH_4HCO_3 and eluted as shown in Figure 5. Pools were made as indicated, dialyzed against 0.2 M NH_4HCO_3, and lyophilized. A similar separation of fragments could be obtained by reduction and alkylation of F2, but 0.1 M mercaptoethanol was required as the reducing agent. Sequence analysis of F3-A revealed the presence of two peptides. The major sequence represented the fragment beginning at residue 79 and was contaminated (~20%) by the peptide beginning at position 175 in the constant region. F3-B was found to be a single fragment beginning at position 79. F3-C contained the amino-terminal (1–32) and the small constant region peptide beginning at position 175 and ending at 214. Since F3-B had been identified as the fragment containing the remainder of the variable region, 300–350 nM of this fragment was subjected to 30 cycles of degradation and the resulting sequence is given in Figure 1. This fragment is easily placed by homology (Kabat et al., 1976) as constituting the remainder of the variable region and no overlap was determined for positions 78 and 79.

Discussion

In the present communication we report the complete variable region sequences of κ chains from three β(1 → 6)-D-galactan binding myeloma proteins. These three are members of a group of seven BALB/c myeloma proteins exhibiting this binding specificity (Potter, 1977). The high degree of homology previously observed in the amino terminus of these chains (Rudikoff et al., 1973) was found to be maintained throughout the first 108 amino acids. As can be seen in Figure 1, X-24 and T-601 are identical for their first 99 residues but then differ at positions 100 and 106. X-44 differs from the preceding two at position 96 in the third light chain CDR (L3). This protein also differs from the X-24, T-601 pair at position 100 but is identical with X-24 at position 106. These differences are especially intriguing in light of recent DNA sequence studies which indicate that the λ (Tonegawa et al., 1978) and

κ (Seidman et al., 1978) variable regions end at amino acids 98 and 97, respectively.

If the results of the κ chain DNA sequencing study are applicable to all κ chains, then the Trp (X-44)-Ile (X-24 and T-601) substitution at position 96 would be the only variable region difference among these three proteins and X-24 and T-601 would, in fact, have identical variable regions. This would be the first example of the occurrence of completely identical κ chain variable regions of independent origin. The Trp-Ile interchange at 96 is intriguing in that a change would have to occur in all three codon bases to generate the observed amino acid difference. If this substitution were a somatic event, an appropriate mechanism must be available for mutating three adjacent bases or for deleting and inserting three base codons. The alternative explanation would be that at least two germ line genes are required to code for these structures and that these genes differ by at least one codon as reflected in the primary amino acid structure. Another interesting feature of these three variable regions is that the amino acids at framework positions 40 (Ser), 46 (Pro), and 47 (Trp) are unique and have not been found in other human or mouse κ chains (Kabat et al., 1976). An examination of the previously reported three-dimensional structure of M603 (Segal et al., 1974) revealed that none of these substitutions would alter the framework structure or folding of the light chain.

The interchanges observed at positions 100 and 106 could be located in the constant region or in other DNA segments. It is unclear at this point whether the constant region is coded for by a contiguous DNA sequence corresponding to amino acids 98 to 214 or if other intervening sequences exist as found between the variable and constant region DNA (Tonegawa et al., 1978; Seidman et al., 1978). Thus, the DNA coding for amino acids beginning at position 98 and extending toward the C terminus may not be contiguous with the major portion of the constant region DNA. The finding of a different amino acid in each protein at position 100 (Gly, Ser, and Ala) (Figure 1) suggests the possibility that multiple genes may exist for this segment of the molecule. This interpretation is based on the following observations. Only 12 murine κ chains have been sequenced at position 100 (Kabat et al., 1976) which, although only a small and probably nonrandom sample, have revealed the presence of four amino acids at this position Gly (8), Ala (1), Ser (2), and Gln (1). The occurrence of Gly, Ser, and Ala at this position in the antigalactan proteins is thus unlikely to be a random event. The Gln at position 100 found in κ chains from anti-azophenylarsonate antibodies (Capra et al., 1977) is also the most common amino acid found at this position in human κ chains. Furthermore, in a fourth galactan binding protein, J539, we have also found Ala at position 100 and Leu at 106 (Rao, D. N., & Rudikoff, S., unpublished data). Thus, Ala (100) and Leu (106) (Figure 1) become linked amino acids found in J539 and T-601 whereas Ile is found at 106 in X-44 and X-24 associated with either Gly or Ser at 100.

The minimal variation observed in the anti-galactan light chains strongly resembles that seen in λl chains studied by Weigert & Riblet (1976). Of 18 λl chains sequenced to date, 11 are identical in the variable region while the remaining seven differ by no more than three amino acids with all substitutions occurring in the CDR. Since λ chains comprise only about 3% of the normal BALB/c light chain pool (McIntire & Rouse, 1970) and the pattern of variation is similar to that of the κ chain isotype containing the anti-galactan light chains, we would suggest that the λl isotype may be roughly equivalent to a minimal variation κ isotype as exemplified by the proteins described in this paper.

The question we are now confronted with is, "How many κ isotypes exist and are multiple germ line genes required to code for given isotype groups?" Amino-terminal sequence studies have revealed to date 28 different isotypes (Hood et al., 1970; Appella & Inman, 1973; Potter et al., 1976a), each of which would presumably require at least one germ line gene. Estimates of the total number of κ isotypes now exceed 100 (Weigert & Riblet, 1976). McKean et al. (1978) have analyzed 8 κ chains from a single isotype group ($V_{\kappa 21}$) which, with the exception of two which have Asn instead of Asp at position 1, are identical for the first 23 amino acids. Two of these proteins A22 and M63 ($V_{\kappa 21B}$) are identical except at position 96 where a Trp-Tyr interchange occurs. A22 and M63 share a series of nine linked amino acids occurring in both framework and CDR which are not found in the other members of the same isotype group ($V_{\kappa 21A,C,D, \text{ and } E}$). It seems quite unlikely that so many identical linked amino acids would be generated by somatic mutation from a common precursor germ line gene in two individual animals in parallel; thus, one germ line gene would be required to code for these two proteins. Two additional subdivisions of this isotype group, $V_{\kappa 21A}$ and $V_{\kappa 21C}$, can further be defined, each of which has two members which share linked amino acids and would presumably be coded for by separate germ line genes. The remaining two proteins in the $V_{\kappa 21}$ group are sufficiently different from the other members to suggest that they could also be coded for by additional germ line genes. If each of these subsets, $V_{\kappa 21A,B,C}$, was coded for by a germ line gene, then variation within any subset would be restricted to CDR. It thus appears that multiple germ line genes would be required to code for the closely related sequences of this isotype group.

Only one other example is currently available on the structure of κ chains from antigen binding myeloma proteins which are members of the same isotype. We have previously reported the comparison of light chains from two phosphorylcholine binding myeloma proteins (Rudikoff & Potter, 1978). Eight substitutions were found in these proteins, only one of which occurred in a CDR. It is unclear whether these proteins are somatic derivatives or are coded for by separate germ line genes.

The patterns of variation observed among related immunoglobulin heavy chains display additional facets to those seen in the light chains. Extensive data are currently available for heavy chains from phosphorylcholine and levan binding myeloma proteins. Four heavy chains from phosphorylcholine binding myeloma proteins have been reported to be identical throughout the variable region, and a fifth differs by a single amino acid in the third CDR (Hood et al., 1976). This pattern of minimal variation resembles that observed for λl, the $V_{\kappa 21}$ subsets, and the anti-galactan light chains. Sequence analysis of other heavy chains from anti-phosphorylcholine proteins has, however, revealed additional variation patterns. When M603 was compared with the group of identical heavy chains, the framework portions of the molecules were found to be the same, but substitutions were found in the second and third CDR as well as a deletion in H3 (Rudikoff & Potter, 1974). A seventh chain, from M167, was found to have substitutions in the framework, the second and third CDR, and a two residue insertion in H3 (Rudikoff & Potter, 1976). The differences observed in the cases of M603 and M167 suggest that as in the $V_{\kappa 21}$ subsets additional germ line genes may be required or novel somatic mutation mechanisms are operative in generating these structures. The second group of related heavy chains analyzed are from levan binding myeloma proteins. Among four of these chains completed to date, a total of six amino acid substitutions were observed, all of which occurred in the framework portion of the variable region (Vrana et al.,

1978). While this variation is indeed minimal, it does not occur in CDR as in the systems noted above. It thus seems unclear at this time as to whether the DNA coding for light and heavy chains has diversified in the same manner or is subject to the same mutational pressures.

In summary, we have found that the κ chains from two galactan binding myeloma proteins have identical variable region sequences and a third differs by a single amino acid located in the third CDR. This minimal variation is similar to that observed for λI chains and for subsets of the $V_{\kappa 21}$ isotype group. The $V_{\kappa 21}$ isotype group may be coded for by as many as five germ line genes based on the structural studies described above. In the case of the anti-galactan light chains we favor the conclusion that at least one germ line gene is required to code for these structures. Analysis of other members of this isotype group may, as in the case of $V_{\kappa 21}$, identify further subsets which require additional germ line genes. Identification of amino acids which occur in high frequency or in a linked fashion in the framework segment following the third complementarity determining region suggests that multiple germ line genes are required to code for this area of the molecule.

Note Added in Proof

Since submission of this manuscript, Brandt and Jaton have also reported identical κ chain variable regions from two rabbit antibodies ((1978) *J. Immunol. 121,* 119).

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