SEQUENCE DIVERSITY WITHIN A SUBGROUP OF MOUSE IMMUNOGLOBULIN KAPPA CHAINS CONTROLLED BY THE *IgK-Ef2* LOCUS*

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Kappa chains comprise >90% of the light chains of mouse immunoglobulins (1, 2). These chains show remarkable diversity of amino acid sequence, and this is now known to be the result of several genetic factors, including (*a*) multiplicity of germ-line genes coding for the variable (V)[1] region (residues 1–95) (3–8), (*b*) combinatorial association of different V regions with four different J regions (residues 96–108) (5, 9, 10) and (*c*) junctional diversity resulting from variation generated at the point of recombination of V and J regions (residues 95–96) (11–13). The magnitude of this genetically programmed diversity is great because each of the factors may potentially act in a multiplicative fashion. In addition to this programmed diversity, an additional order of magnitude of variation could occur as a result of spontaneous somatic mutation acting at the level of the lymphocyte cell population (14).

It would be possible to assess the importance of somatic mutation in generating kappa chain sequence diversity if one could sequence a large number of kappa chains that were all coded by a single V-region gene, Vk. We have attempted to approach this problem by characterizing a group of light chains that are structurally closely related and which are controlled in a mendelian fashion (15, 16). The light chains, which are derived from a series of BALB/c myeloma proteins, all cofocus precisely with a pair of prominent polymorphic bands observed in normal light chain isoelectric focusing (IF) profiles. The normal light-chain IF bands in question have been shown to be controlled by a chromosome 6 locus designated *IgK-Ef2* (15). A second, similar locus, *IgK-Ef1*, has been shown to control other groups of closely related light chains observed as distinct bands in normal light-chain IF profiles (17). One of these groups has recently been shown to represent a unique group of kappa chains having an N-terminal serine residue (18). Because both the *IgK-Ef1* and *IgK-Ef2* loci seem to control several sets of normal light-chain IF bands, it is possible that they may represent distinct blocks of V region of kappa chain (Vk) genes that have been maintained in the genome of certain mouse strains. That the mouse Ck gene as well

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[1] *Abbreviations used in this paper:* cdr, complementarity-determining; Ck, the constant region of the kappa chain; fr, framework; HPLC, high-performance liquid chromatography; IF, isoelectric focusing; IOBA, iodosobenzoic acid; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; V, variable, Vk, V region of the kappa chain.

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as certain Vk genes have been physically located on chromosome 6 (19, 20) would support a structural gene basis for the polymorphisms.

In this study, we have sequenced a group of four BALB/c light chains corresponding to bands controlled by the *IgK-Ef2* locus. We have also sequenced two closely related kappa chains derived from NZB mice, an *IgK-Ef2*^{b} strain. The sequences indicate that the BALB/c light chains fall into a single V-region subgroup showing limited sequence diversity. The NZB proteins represent a closely related subgroup distinct from the BALB/c subgroup. The results are discussed in terms of the nature of the genetic polymorphism and the origin of intrasubgroup diversity.

Materials and Methods

Myeloma Proteins. BALB/c and CAL-20 myeloma tumors TEPC-817, generation 6 (G6), TEPC-821 (G6), FLOPC-1 (G15), and TEPC-105 (CAL-20) (G8) were generously provided by Dr. Michael Potter, National Institutes of Health, Bethesda, Md. These tumors have been maintained at Litton Bionetics, Kensington, Md. under National Cancer Institute, Bethesda, Md. contract NO1-CB-92142 to Dr. Michael Potter. Ascites fluids were generated at the Université de Sherbrooke, Sherbrooke, Quebec, Canada by intraperitoneal inoculation of pristane-primed BALB/cByJ and CAL-20 mice (21). Ascites fluids from NZB tumors PC 2205 and PC 2567 were generously furnished by Dr. Martin Weigert, Institute for Cancer Research, Philadelphia, Pa. Myeloma proteins were purified by precipitation three times of twofold-diluted ascites fluid with an equal vol of 3.5 M ammonium sulphate, pH 7.3 (22). Light chains were isolated after partial reduction and alkylation of the immunoglobulin with iodoacetamide by gel filtration on columns of ACA 34 (LKB Produkter AB, Stockholm, Sweden) equilibrated with 3 M guanidine hydrochloride and 0.2 M ammonium bicarbonate.

Enzyme Digestion, Fragment Purification, and Peptide Mapping. Native light chain (10 mg/ml) was digested with staphylococcal V8 protease (23) (Miles Research Laboratories) in 0.05 M ammonium bicarbonate, 0.2% sodium dodecyl sulphate at an enzyme:light chain ratio of 1:25 for 24 h at 37°C. The digest was lyophilized and fractionated on Sephadex G-75 equilibrated with 8 M urea, 0.05 M acetic acid (Fig. 1 a). The major peak (B, Fig. 1 a) was then desalted on a column of Bio-Gel P2 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 1 M acetic acid. The material was then completely reduced and alkylated using [^{14}C]iodoacetamide and dialyzed against 0.05 M ammonium bicarbonate using boiled Spectraphor 3 tubing (Bausch and Lomb, Inc., Rochester, N. Y.). This resulted in precipitation of the fragment V8-1 (Fig. 2). The supernate, containing peptides V8-2 and V8-3, was fractionated on DEAE-Sephacel using a gradient of ammonium bicarbonate from 0.05 to 0.5 M (Fig. 1 c). The fragment V8-1 was succinylated, digested with trypsin, and fractionated on DEAE-Sephacel using a gradient of ammonium bicarbonate from 0.05 to 1 M (Fig. 1 b). Tryptic and chymotryptic digestion of completely reduced and alkylated light chain was carried out for 3 h at 37°C at an enzyme: light chain ratio of 1:50. Peptides were isolated from preparative (5 mg) peptide maps by cutting out spots staining with 0.02% ninhydrin in acetone (25). Peptide mapping on Whatman 3 MM paper (Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England) combined electrophoresis at pH 3.5 (2.7 kV, 90 min) followed by descending chromatography in butanol/acetic acid/water/pyridine (15:3:12:10). Tryptic and chymotryptic peptides identified in all proteins are indicated in Fig. 2. Amino acid compositions were determined on a Technicon TSM-1 amino acid analyzer (Technicon Instruments Corp., Tarrytown, N. Y.) (26).

Fragmentation With Iodosobenzoic Acid (IOBA) (27). Completely reduced and alkylated light chains were dissolved in 80% acetic acid-4 M guanidine-hydrochloride. An equal weight of IOBA (Pierce Chemical Co., Rockford, Ill.) was then added and the reaction was allowed to proceed for either 3 or 24 h at room temperature in a brown bottle. After terminating the reaction by the addition of an excess of mercaptoethanol, the mixture was diluted twofold with water, centrifuged, and the supernate was fractionated on a column of Sephadex G-50 equilibrated with 8 M urea-0.5 M propionic acid. Individual peaks were pooled and subsequently fractionated on columns of SE-Sephadex equilibrated with 8 M urea-0.005 M formic acid buffer, pH 3.0, using gradients of KCl, 0–3 M (28). Cleavage for 24 h led to extensive

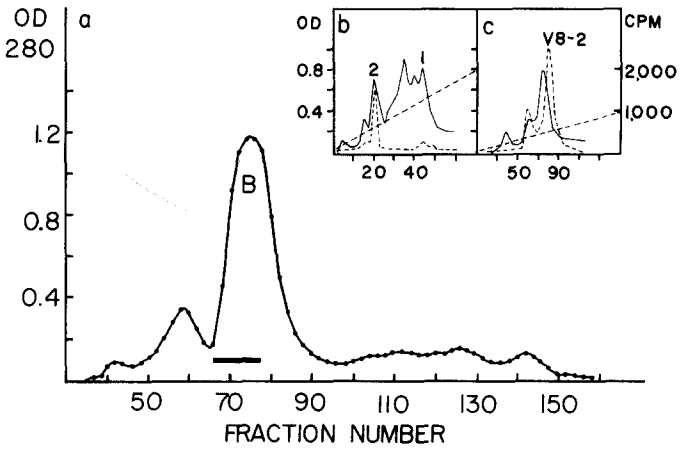

FIG. 1. (a) Fractionation of a staphylococcal V8 protease digest of 50 mg of the partially reduced and alkylated light chain of TEPC-821 on Sephadex G-75 in the presence of 8 M urea-0.05 M acetic acid. (b) Fractionation of a trypsin digest of the succinylated fragment V8-1. V8-1 represented the insoluble material obtained upon complete reduction and alkylation of peak B (Fig. 1 a). The separation was performed on a column (0.6 × 8 cm) of DEAE-Sephacel equilibrated with 0.01 M ammonium bicarbonate. A linear gradient of ammonium bicarbonate 0.01–1.0 M (total vol 80 ml) was used for elution. (c) Fractionation of the soluble peptides obtained after complete reduction and alkylation of the material from peak B (a). Column conditions were as in (b) except for the salt gradient, which was from 0.01 to 0.5 M in this case.

hydrolysis at tyrosine as well as tryptophan residues (29) and fractionation was difficult. At least one useful fragment (IOBA-3, Fig. 2) resulting from tyrosine cleavage was purified. A less complex mixture was obtained in the 3-h IOBA digests, and these were used for the isolation of fragments IOBA-1 and IOBA-2.

Sequenator Procedures. Automated sequencing of reduced and alkylated intact light chains and fragments was performed using a modified Illitron model 9001 sequenator with a 0.33 M quadrol program. For peptides and fragments, the sequenator was pre-run for three cycles with 4 mg Polybrene plus 100 nmol of glycylglycine (30). The samples from the sequenator were converted manually using 25% trifluoroacetic acid (TFA) for 10 min at 60°C. After drying the TFA, the samples were dissolved in 0.1 ml methanol:acetonitrile (50:50) and analyzed by high-performance liquid chromatography (HPLC). HPLC separation of phenylthiohydantoin (PTH) amino acids was carried out on a Waters (Waters Associates, Milford, Mass.) instrument using Dupont CN-columns (31). In some instances, samples were identified by amino acid analysis after alkaline dithionite hydrolysis (32).

Results

Sequencing of the Light Chains. The strategy used in sequencing the light chains is summarized in Fig. 2. Because glutamic acid occurred at only three positions in the V region (positions 79, 81 and 105; numbering according to Kabat et al. [3]), cleavage of the native light chain with staphylococcal protease V8, an enzyme specific for glutamyl peptide bonds (23), resulted in the formation of a large disulphide-bonded V-region fragment that could be isolated on Sephadex G-75 in the presence of urea (Fig. 1). Complete reduction and alkylation of the fragment with iodoacetamide resulted in precipitation of a mixture of large N-terminal fragments (residues 1-79, 1-81, and 1-105). The soluble fraction contained a fragment corresponding to residues 81–105. This was purified (Fig. 1 c) and could be sequenced up to and including the C-terminal glutamic acid residue (Fig. 3). The insoluble fraction was succinylated

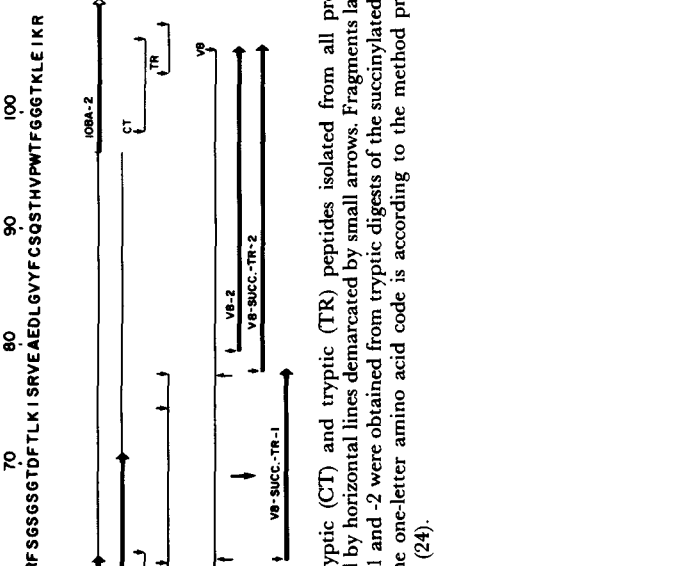

FIG. 2. Sequencing strategy used for the variable region of Vk-1A and Vk-1B light chains. Heavy horizontal arrows indicate regions sequenced using the sequenator. Sequencing was greatly facilitated by the use of staphylococcal V8 protease (V8) which permitted isolation of an entire V-region fragment (V8-1) and fragments covering the third hypervariable region and J region. IOBA-1, -2, and -3 are fragments isolated from IOBA cleavage of the intact light chain. Chymotryptic (CT) and tryptic (TR) peptides isolated from all proteins are indicated by horizontal lines demarcated by small arrows. Fragments labeled V8-Succ-Tr-1 and -2 were obtained from tryptic digests of the succinylated fragment V8-1. The one-letter amino acid code is according to the method proposed in reference (24).

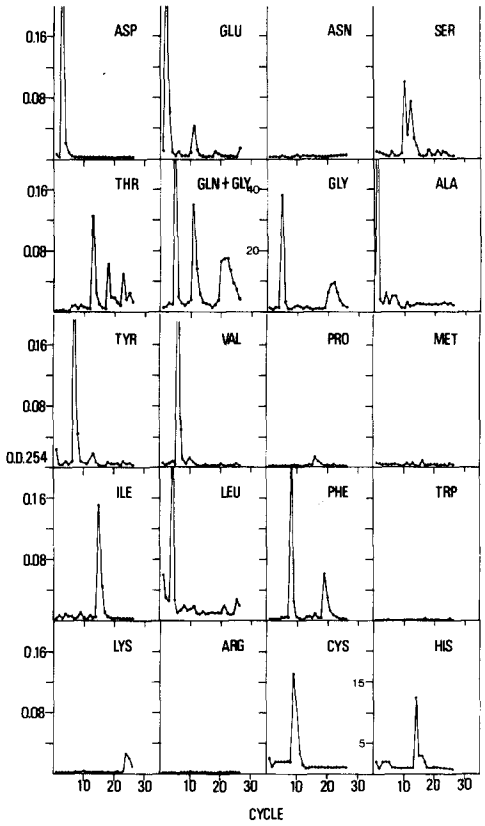

FIG. 3. Recoveries of PTH-amino acids in the automated degradation of fragment V8-2 obtained from the light chain of FLOPC-1. Because of incomplete resolution of PTH-glutamine (PTH-Gln) and PTH-glycine (PTH-Gly), data is included for the recovery of glycine after alkaline dithionite hydrolysis. The PTH-derivatives of dehydro-serine, dehydro-threonine, and *S*-carboxymethylamido cysteine were determined by monotoring the effluent of the HPLC at $A_{313\ nm}$. Other PTH amino acids were followed at $A_{254\ nm}$. The data for histidine are from the recovery of histidine after alkaline dithionite hydrolysis. The location of cysteine was also determined by the location of the ^{14}C radioactivity, as the fragment was in the [^{14}C]carboxymethylamido form. The sequence deduced from this data corresponds to residues 80–105 (Fig. 4).

and subjected to cleavage with trypsin. The resulting fragments were fractionated on DEAE-Sephacel (Fig. 1 b) and subjected to automated sequencing. Tryptic fragments containing residues 25–54, 62–77, and 78–105 were isolated in this way. The iodosobenzoic acid cleavage fragment beginning at residue 36 was sequenced for 24 cycles over the region 36–60. An additional fragment resulting from IOBA cleavage at tryosine 49 gave a sequence over residues 50–68, linking the sequences obtained with the other fragments. Complete sequencing of all proteins was done over residues 1–44 and 78–105. The internal residues, 45–77, were completely sequenced as described only for proteins TEPC-817 and FLOPC-1. In the remainder of the proteins, the region of residues 45–77 was determined by composition and mobilities of tryptic and chymotryptic peptide only.

Sequence Diversity in Ef2-related Light Chains. Comparison of the four BALB/c light

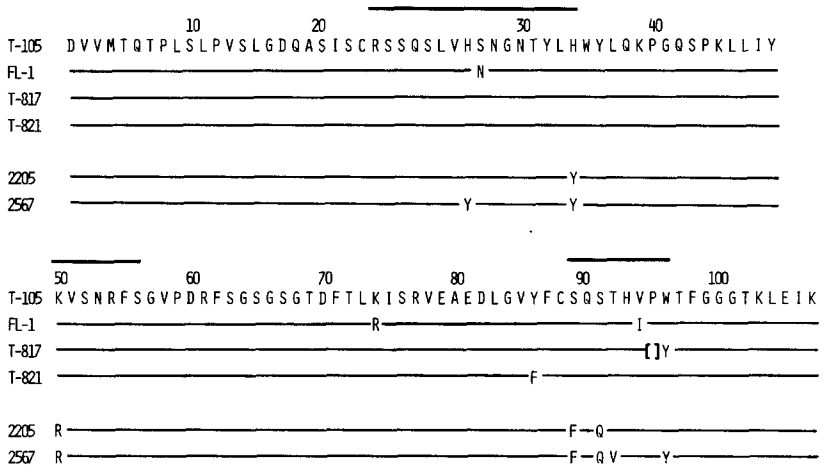

FIG. 4. Amino acid sequences of the variable regions of the BALB/c myeloma light chains TEPC (CAL 20)-105, FLOPC-1, TEPC-817, and TEPC-821 and NZB myeloma light chains PC 2205 and PC 2567. The single letter code is according to a previously proposed method (24). Numbering and location of cdr regions (horizontal bars over the sequence) is according to Kabat et al. (3).

chain sequences (Fig. 4) indicates they fall into a very closely related subset of kappa chains. By the classification of Potter (33) these sequences represent a subgroup of the Vk1 group, a group that includes the light chain of the dinitrophenyl-binding myeloma protein MOPC-460 (34). The sequences of TEPC-817 and TEPC (CAL-20)-105 are identical up to position 95, where the normally highly conserved proline residue is deleted in TEPC-817. The other two sequences (FLOPC-1 and TEPC-821) differ from the prototype (TEPC-105) sequence by three (two complementarity-determing regions [cdr] and one framework [fr] region) and one (fr) residues respectively.[2] None of the substitutions involves a net change in charge, as expected because the proteins were all selected on the basis of their charge identity in IF.

Strain-specific Sequence Differences. Comparison of the N-terminal sequences of *Ef2*-related BALB/c light chains with sequences available for NZB light chains (26) revealed that two examples of closely related light chains had been observed in NZB myelomas (PC 2205 and PC 2567). Because NZB mice are negative for the *Ef2* marker in normal light chain profiles and no examples of *Ef2*-identical light chains were detected by IF screening of 133 NZB myelomas (16), the existence of these two light chains suggested that NZB mice must carry V genes for a subgroup closely related to the *Ef2* subgroup. We previously showed that light chains of NZB myelomas PC 2205 and PC 2567 differed slightly from the *Ef2* marker bands on IF (16). Sequencing of the V regions of the NZB proteins has revealed that they differ from the BALB/c proteins by only four residues (Fig. 4). The presence of a tyrosine in place of histidine at position 34 is sufficient to explain the slight anodal charge shift in the NZB proteins. The two NZB proteins themselves differ by two residues in the V region, one of these is a tyrosine-histidine substitution. This would explain the slight IF difference we observed between the two proteins (16).

[2] The fr and cdr regions are as defined by Kabat (3).

Discussion

Complete V-region amino acid sequences of four BALB/c light chains corresponding to normal light chain IF-bands controlled by the *IgK-Ef2* locus indicate that the light chains differ from one another by one to three residues (Fig. 4). The question that arises is whether they may be all coded by a single Vk gene. It seems likely that at least two of the proteins, TEPC-817 and TEPC (CAL-20)-105 are coded by the same V gene, because the only difference between the two proteins is the deletion of proline 95 in the TEPC-817 sequence. This seems likely because of deletion of codon 95 at the time of joining V-817 and J4. This is the first instance of a deletion at this position, but it not unexpected in view of the apparent sloppiness of V-J joining (13). Other examples of this phenomenon have resulted in the deletion of residue 96 (in PC 8701 [11]) and the insertion of an extra residue between position 95 and 96 (in PC 7132 [5]). More recently, an out-of-phase V-J joining has been shown to result in the formation of a cryptic light chain gene (13).

Although TEPC-817 and TEPC (CAL-20)-105 light chains are probably derived from a single V gene, the other two light chains, FLOPC-1 and TEPC-821 differ from the prototype sequence by three (one fr, two cdr) and one (fr) residues, respectively. The occurrence of two substitutions in the cdr regions of FLOPC-1 can be attributed to somatic mutation of the type seen in mouse lambda chains (14). Differences in the framework region however cannot be accommodated in the classical somatic mutation-selection model. Such differences are generally attributed to the existence of additional germ-line genes. A less likely possibility is that they represent residual heterozygosity within the BALB/c population. Evidence that germline-coded Vk genes may differ by as few as two framework residues is found in kappa subgroups Vk-21E and Vk-21F. Here, repeat substitutions of two framework residues occur in the NZB light chains PC 2485 and PC 4039 (Vk-21F) when compared with PC 7175 and PC 6684 (Vk-21E) (5). In the present work, we are unable to say whether the framework substitutions observed represent additional Vk genes because no repeat occurrences have been found. Sequencing of additional *Ef2*-related light chains is under way to further document the diversity within this subgroup. We propose to designate the subgroup Vk-1A based on the original sequence classification of Potter (33).

Sequencing of two closely related light chains from NZB myelomas PC 2205 and PC 2567 indicates that these two light chains are derived from a different Vk gene. These light chains share four identical substitutions compared with the Vk-1A proteins of BALB/c. That NZB mice and other *IgK-Ef2*^{b} strains (15) fail to express light chains of the Vk-1A subgroup is most easily understood in terms of a loss of the Vk-1A gene or gene cluster in these strains. It is also possible that the Vk-1A gene(s) are present but not expressed because of regulatory effects (e.g., at the level of translocation). Regulatory influences directed at a specific Vk subgroup at the cellular level are difficult to imagine because it is likely that Vk-1A light chains occur in a variety of functional combinations with different heavy chain partners. That the Vk-1A and Vk-1B subgroups differ by four substitutions suggests that they may not be true alleles, but instead represent different structural gene loci. If this is the case, it is possible that BALB/c may possess both the Vk-1A and Vk-1B genes. However, we have so far found no examples of Vk-1B light chains in a screening of >200 BALB/c myelomas.

Resolution of the question of whether the subgroup is coded by a single-Vk-1A gene or a series of virtually identical genes repeated in tandem awaits the results of DNA studies now in progress. That the light chains correspond to a single pair of IF bands that behaves in a mendelian fashion would tend to favor a single-gene origin for the subgroup. If the sequences are derived from a single Vk-1A gene, it would mean that the framework substitutions observed must be a result of somatic mutation either before or during the myeloma induction or propagation. Substitutions outside of the cdr regions may not be completely neutral because they could conceivably affect the antigen-binding site or the idiotypic character of the molecule by altering the three-dimensional structure of the V region. It is clear from the very existence of a great variety of V-region subgroups that selection for framework diversity must occur in evolution. Similar forces could also act at the somatic level.

Summary

We previously showed that a chromosome 6 locus, *IgK-Ef2*, controls a pair of prominent bands in normal mouse light-chain isoelectric focusing profiles. Screening of myeloma light chains derived from BALB/c mice (an *IgK-EF2*^{a} strain) led to the identification of seven light chains cofocusing with the polymorphic bands controlled by *IgK-Ef2*. Complete sequencing of the variable (V) regions of four of the light chains indicates that they are all members of the same subgroup (Vk-1A) and they differ from one another by 1–3 substitutions. One of the proteins differs from the prototype V-region sequence only in the deletion of a single residue at position 95 immediately preceding the J region. The other two differ from the protoype V region by 3 (two framework [fr], one complementarity-determining [cdr]) and one (fr) residues, respectively. Complete V-region sequences of two closely related light chains derived from NZB mice (an *IgK-Ef2*^{b} strain) indicate the NZB proteins are derived from a distinct Vk gene (Vk-1B), differing by four substitutions from the Vk-1A sequence. The results suggest that the *IgK-Ef2* polymorphism may be a result of, at least in part, the loss of the gene(s) coding for the Vk-1A subgroup in *IgK-Ef2*^{b} strains of mice. The nature of the sequence diversity found in the Vk-1A subgroup indicates that either it is coded by a repeated series of virtually identical genes or that somatic mutation of a single Vk-1A gene may give rise to substitutions in framework as well as cdr regions.

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