STRUCTURE AND FUNCTION OF THREE NOVEL MHC CLASS I ANTIGENS DERIVED FROM A C3H ULTRAVIOLET-INDUCED FIBROSARCOMA

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The class I antigens encoded within the MHC of the mouse are cell surface glycoproteins that play an essential role in immune recognition and regulation. CTL exhibit an absolute requirement for associated recognition of foreign antigens in the context of self MHC components (1). In addition, CTL can recognize non–self MHC molecules. This specific recognition of non–self MHC, known as allore cognition, is the cellular basis of the well known phenomenon of allograft rejection.

Class I antigens also appear to be involved in the immune elimination of certain tumors (2, 3). Many tumors are highly immunogenic and are rejected when transplanted into normal, histocompatible mice (2–6). The ability of such tumors to arise in the autochthonous host might therefore represent the consequence of a compromised immune system that cannot limit neoplastic progression. In fact, many carcinogenic treatments, such as UV irradiation, are also immunosuppressive (5), and this might contribute to the outgrowth of otherwise immunogenic neoplastic cells. Consequently, ~75% of the tumors that arise in UV-treated mice are rejected in normal syngeneic recipients but progress in UV-irradiated animals (5, 6). Interestingly, the immune suppression associated with UV irradiation appears to be specific for tumors arising in UV-irradiated hosts (7) and can be mediated by specific Ts (8). To understand the complex biology of these tumors, it is necessary to identify and characterize the molecules responsible both for their immunogenicity and for their ability to escape the immune system in UV-treated animals.

One UV-induced tumor that has been extensively studied is the C3H fibrosarcoma, 1591 (5). It has recently been shown (9, 10) that 1591 expresses at least three novel class I antigens not normally found on C3H tissue. These novel antigens have been identified by mAbs derived from normal C3H mice immunized with the tumor and are also recognized by allospecific anti-H-2 mAbs (9, 10)
Certain tumor variants lacking the novel antigens can grow progressively in immunocompetent hosts, suggesting that these molecules may play an important role in the biology of this tumor (11). The isolation of the genes encoding the novel antigens, termed A149, A166, and A216, has been previously described (12). Since these genes account for several of the polymorphic restriction fragments observed in the class I genes of 1591 when compared with C3H by genomic Southern blot analysis, we have previously proposed (12) that these genes were generated by recombination events among the endogenous class I genes of C3H. To determine the origin of these novel genes and the role of their products in the neoplastic process, it is necessary to characterize their structure and examine the extent to which each molecule can be recognized by the immune system.

In this report, we present the complete DNA sequence of these three novel class I genes, and describe in detail the recognition of their products by alloreactive and tumor-specific CTL. We find that these genes are highly homologous to those of previously characterized class I antigens, such as H-2Kk (13) and H-2Ld (14), and that the structural conservation seen among these gene products is reflected in their functional homology as well. Since the class I diversification events by which these novel tumor genes were generated probably parallel events that occur in the germline, the identification and analysis of the parental C3H genes from which they were derived might be relevant to the study of class I evolution. Furthermore, the discrete pattern of structural and functional homology seen among A149, A166, and H-2Ld suggests that functional analysis of these novel H-2L products should provide significant insight not only into the role that these molecules played in the progression of the 1591 tumor, but also into the structural basis of T cell recognition.

Materials and Methods

Materials. Restriction endonucleases were obtained from New England Biolabs, Beverly, MA. *Escherichia coli* DNA polymerase Klenow fragment was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. *E. coli* strain JM103, phage M13mp18, and mp19 were the gift of Dr. Chuck Staben (Department of Biochemistry, University of California, Berkeley). Oligonucleotide primers homologous to class I sequence were synthesized by Lynn Williams at the University of Southern California Medical Center, and by Bruce Malcolm at the Department of Biochemistry, University of California, Berkeley.

Cell Lines and Animals. The C3H origin of the 1591 tumor has been previously documented (9, 10, 12). The 1591 tumor and the 28" tumor variant (9) were the kind gift of Dr. Hans Schreiber (Department of Pathology, University of Chicago). L cell transfectants were generated previously (12). Mice were bred and maintained in the colony at the University of Texas Health Science Center, Dallas.

DNA Sequence Analysis. The genes were sequenced in parallel by the dideoxy chain termination method (15) using a set of 40 synthetic oligonucleotide primers complementary to class I sequences. We chose primers that hybridize at 200–250 bp intervals in both orientations, according to the DNA sequence of the *H-2D* gene (16). Up to 350 bp were read per sequencing reaction on 5% urea/7% polyacrylamide gels.

Generation of Cytotoxic T Lymphocytes. To generate CTL activity against the 1591 tumor or L cells transfected with tumor-derived class I genes, (C3H × dm2)F1 animals were inoculated intraperitoneally with 10⁵ tumor or transfected L cells. ~3 wk later, spleens were removed from the primed animals and these cells were cultured in vitro with either x-irradiated (3,000 rad) tumor cells, L cells transfected with the tumor genes, or
**Table 1**

| Antibodies | α1  | α2  | α3  |
|------------|-----|-----|-----|
| 34-1-2     | +   | -   | +   |
| 34-4-20    | +   | -   | -   |
| CP3F4      | +   | +   | -   |
| 30-5-7     | +   | +   | +   |
| CP28       | +   | -   | +   |
| 28-14-8    | +   | +   | +   |

Summary of the class I specificities displayed on the UV tumor 1591. Specificities and tentative domain assignments of antibodies 34-1-2, 34-4-20, 30-5-7, and 28-14-8 were previously determined (19-22). CP28 and CP3F4 are 1591-specific mAbs (9), which have been mapped to the α2 domain (M. McMillan, University of Southern California, Medical Center, personal communication). Reactivities were previously measured by cell-binding RIA of 1591 tumor cells or L cell transfectants (12). 34-1-2 reactivities are unpublished data (H. Stauss). +, strong reactivity; ±, weak reactivity; -, no reactivity.

BALB/c spleen cells. 5 d later, the cultures were tested for cytolytic activity against 51Cr-labeled target cells. CTL clones were generated as described previously (17). All of the clones are specific for H-2Ld and were derived from either CBA anti-A.AL or (C3H × dm2)F1 anti-BALB/c cultures. The clones are maintained in the presence of rat Con A supernatant and irradiated spleen feeder cells.

Target Cells. The target cells consisted of either L cells expressing transfected class I genes or 1591 tumor cells. Targets were labeled with 51Cr and between 2 × 10^5-10^6 cells were added to wells containing effector cells. After 4 h of incubation, the culture supernatants were tested for radioactivity. Specific release = (experimental release - spontaneous release)/maximum release.

CTL Competition Assay. This assay has been described previously (18). Briefly, varying numbers of unlabeled L or tumor cells were added to a mixture of effector and labeled target cells in the cytolytic assay. Data are presented as: % inhibition = [1 - (specific release in the absence of inhibitor cells - specific release in the presence of inhibitor cells)/specific release in the absence of inhibitor cells] × 100.

Results

Expression of the Novel Antigens by L Cell Transfectants, and Serological Characterization of Novel Gene Products. The 1591 tumor is recognized by several allo- and tumor-specific mAb unreactive with products expressed on normal C3H tissues (9, 10). As summarized in Table 1, these include the syngeneic anti-1591 mAbs CP28 (9) and CP3F4 (9) and the H-2L crossreactive antibodies 34-1-2 (19, 20), 30-5-7 (19, 21), and 28-14-8 (19, 21). Genomic λ clones A149, A166, and A216 were isolated from a 1591 genomic library on the basis of crosshybridization to the class I-specific cDNA clone, pH-211a, and identified as encoding the 1591 tumor-specific epitopes by transfection into C3H L cells and RIA screening of transfected with a panel of tumor-reactive antibodies, as previously described (12). The serological reactivities of the individual novel class I gene products, as
well as other related class I antigens, are summarized in Table I. All of the allo- and tumor-specific class I serological reactivities seen on the 1591 tumor can be accounted for by the reactivities of the three individual novel class I antigens. We have described two of these antigens as H-2L-like because they are recognized by at least two mAbs crossreactive with the H-2Ld antigen from BALB/c. A149, A166, and H-2Ld share the determinants recognized by the mAbs 28-14-8 and 34-1-2. In addition, both A166 and H-2Ld are recognized by the antibody 30-5-7. The antibody 34-4-20 (22), on the other hand, recognizes neither H-2L-like tumor antigen, although it recognizes both H-2Ld and H-2Dd. The A166-specific mAb, CP3F4, crossreacts with H-2Kd, but it recognizes neither A149 nor H-2Ld transfectedants. The product of the A216 gene is identified by the anti-1591 mAb CP28. In addition, expression of the A216 and A149 products in BALB/c 3T3 cells indicates that these C3H-derived antigens retain several H-2b haplotype reactivities (data not shown).

**DNA Sequence of the Novel Class I Genes Derived from the 1591 Tumor.**

The nucleotide sequence of genes A149, A166, A216, and the H-2Ld clone λ27.5 (14) were determined using the sequencing strategy described in Fig. 1. Briefly, 1–3-kb unique restriction fragments diagnostic for each of the λ clones were subcloned into the polylinker of M13mp18 or mp19. Templates derived from these subclones were sequenced in parallel by the dideoxy-chain termination method, using a set of 40 crosshybridizing synthetic oligonucleotides as primers (see Fig. 1). Sequence differences between the genes could thus be confirmed directly on the same gel.

The DNA sequences of the three novel class I genes are quite homologous to
FIGURE 2. DNA Sequence of A149, A166, and H-2Ld. Unless otherwise specified, the sequence of all three genes is identical. Coding regions are shown as triplets, translated amino acid sequences are shown below. Numbering shown is for H-2Ld. Protein sequence numbering begins at the first glycine in α1 domain. Deletions are represented by -. The Pst I sites defining the 1-kb polymorphic Pst I fragment are underlined.
FIGURE 2.

those of other known class I genes (Figs. 2 and 3). Intron-exon boundaries of A149 and A166 were assigned by comparison with the published sequences of H-2D\(^d\) (16), H-2L\(^d\) (14), and H-2D\(^b\) (23), and A216 by comparison with H-2K\(^k\) (13). All three genes appear to comprise eight exons, the first encoding the leader peptide; three exons encoding the three external domains, \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\); an exon encoding the hydrophobic transmembrane region; and three exons accounting for the cytoplasmic domain of the antigen and the 3'-untranslated trailer sequence. All splice junctions assigned for H-2L\(^d\) and H-2K\(^k\) appear to be represented. Like H-2L\(^d\), A149 and A166 each contain three attachment sites for N-linked glycosylation; like H-2K\(^k\), A216 contains two. The single amino acid substitution of a leucine for a cysteine at residue 121 in the \(\alpha_2\) of A166, does not involve a cysteine normally involved in a disulfide bond.

Since the A216 gene encodes a product recognized by mAbs crossreactive with H-2K\(^k\), and since the A216-specific antibody CP28 crossreacts with H-2D\(^d\), we have compared the DNA sequence of A216 with the published sequences of these class I genes. The overall homology between A216 and H-2D\(^d\) is comparable to that seen between other H-2 genes, such as H-2L\(^d\) or H-2D\(^d\).
FIGURE 3. DNA sequence of the A216 gene. Coding sequences are shown as triplets with the translated amino acid sequences below. Protein sequence numbering begins at first glycine in a1 domain. The Pst I sites defining the 950-bp polymorphic Pst I fragment are underlined.
of A216 and the H-2K\(^k\) from AKR revealed similar homology of 85–90% throughout the 5' ends of these genes. Striking homology of >99%, however, was seen between the 3' ends of these two genes, from a point ~300 bp 5' of exon 4, and continuing for 1,800 bp into 3'-untranslated sequences. A 1-kb polymorphic Pst I restriction fragment not found in the C3H genome (12) maps within this region, suggesting that the 3' end of this gene was generated by a recombination event between two class I genes homologous to H-2K in this region. Preliminary screening of the class I genes from a normal C3H library by restriction mapping and with A216-specific oligonucleotide probes has identified several potential 3' parents, including H-2K\(^k\) (S. Watts, University of California, Berkeley, unpublished results). Thus it is possible that H-2K\(^k\) is itself a parent, and that the minimal degree of divergence, i.e., eight point differences and two small clustered changes, seen between the 3' coding region of A216 and the H-2K\(^k\) sequence might reflect sequencing differences or strain substitutions between the C3H and AKR H-2K alleles. Further sequence analysis of the normal C3H genes will be required to unambiguously identify the parental genes.

Since the molecules encoded by A149 and A166 share serological determinants with each other and H-2L\(^d\), we sequenced these genes in parallel. The complete and revised sequence of H-2L\(^d\), as well as the sequences of the two C3H-derived H-2L-like genes, is presented in Fig. 2. The DNA sequences of the H-2L-like tumor genes are nearly identical to each other, to H-2L\(^d\), and to the corresponding regions of an H-2D\(^b\) cDNA clone (23) for ~3,000 bp from within the large intron until the end of the 166 clone (Fig. 2). Within this region A149 is identical to H-2L\(^d\), and A166 exhibits only two bases that are polymorphic. The only region of these genes for which <99.9% homology is found is within the exons encoding the first and second external domains, a1 and a2, which are believed to be the regions primarily responsible for T cell recognition (24). Within the first 1,000 bp of these genes, A149 and A166 are 99.1 and 98.6% homologous to H-2L\(^d\), respectively.

The two Pst I sites defining one of the polymorphic restriction fragments seen in A149 and A166, but not in the C3H genome, map within the region of near
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FIGURE 4. Comparison of amino acid sequences of tumor antigens with other class I antigens. Sequences are shown from the first and second external domains, α1 and α2. Sequences compared are A149, A166, H-2Ld, H-2Dd (16), H-2Kd (25), H-2K° (13), and H-2Ddmt (26).

identity to H-2Ld at positions 3009 and 4047, suggesting that these H-2L coding sequences exist in unexpressed form within the C3H MHC requiring a specific recombination event to be activated. Since both A149 and A166 contain the same polymorphic Pst I fragment, it is possible that they were generated by duplication and divergence from a single parental recombinant gene. However, analysis of 5'-flanking sequences and restriction enzyme site polymorphisms indicate that these two genes are derived from different 5' parents (data not shown).

Structural Basis for Conserved Serological Determinants on Novel H-2L-like Antigens. The data concerning the serological recognition of these novel gene products can be analyzed in light of the predicted amino acid sequences (Figs. 2-4). The identity between the three L-like antigens in the α3 domain, and the extreme homology between the antigens in the α1 domain are consistent with the shared reactivities with 28-14-8 and 34-1-2, which have been shown to map to the α3 and α1 domains, respectively (20, 21). Since 34-1-2 binds A166 with higher affinity than either A149 or H-2Ld (Table I), it is intriguing that three of the five amino acid substitutions that A166 bears in the α1 domain with respect to H-2Ld, render A166 more homologous to either H-2Kd (25) or H-2Dd, both of which are strongly reactive with 34-1-2.

The lack of reactivity with the α2-specific antibody, 34-4-20 (M. McMillan, University of Southern California, Los Angeles, CA; personal communication), seen for both tumor antigens, correlates with the presence of isoleucine and arginine at positions 95 and 97 within α2 of both molecules. H-2Dd and H-2Ld, which are recognized by this antibody, both have leucine and tryptophan at these two positions. The pattern of reactivity of the antibody, 30-5-7, appears more complicated. Previously, exon-shuffling experiments (21), as well as analysis of the 30-5-7 reactive H-2Ddmt molecule (26) mapped the epitope recognized by
this antibody to the C-terminal two-thirds of the \(a2\) domain. However, it is likely that other regions will be involved in the formation of a functional antigenic epitope, since A149, which is not 30-5-7-reactive, is quite conserved with H-2L\(^d\) and A166, both of which are recognized. For example, the amino acid substitution of a tryptophan for a glycine at position 107 of A149 might somehow interfere with the formation of a functional determinant by the rest of the domain. Oligonucleotide-directed mutagenesis experiments should help resolve this issue.

The tumor-specific mAb CP3F4 recognizes the antigens A166 and H-2K\(^d\). Since the only amino acid residues unique to these molecules are a serine and an alanine at positions 77 and 81 in the \(a1\) domain, it is impossible to identify any specific set of amino acids influencing the epitope recognized by this antibody without additional mutagenesis experiments. Similarly, although the tumor-specific antibody CP28 recognizes both A216 and H-2D\(^d\) (9), the available data do not reveal any obvious pattern of unique structural homology between these molecules.

**Recognition of Novel Antigens by Allospecific CTL.** The 1591 tumor is highly immunogenic and induces a strong CTL response when transplanted into normal syngeneic mice (27, 28). To distinguish the components of the CTL response directed against each of the novel class I antigens, we have tested L cells transfected with the three individual genes for their ability to function as targets for anti-1591 CTL. Since primary in vitro anti-1591 responses could not be detected (data not shown), CTL populations directed against the tumor were generated by immunization of (C3H × H-2\(^{dm6}\))\(F_1\) mice with \(10^7\) 1591 tumor cells. 3 wk later the spleen cells of these mice were placed in culture with irradiated 1591 stimulator cells. After a 5-d culture period, the effector cells were tested against \(^{51}\)Cr-labeled L cell targets, as well as 1591 and the 1591 (28\(^-\)) variant, selected in vitro for the loss of the three novel class I products (11). Both 1591 and L cells expressing each of the novel gene products were recognized and effectively lysed by the anti-1591 CTL (Fig. 5a), as were BALB/c 3T3 cells
transfected with the A216 gene (data not shown). L cells expressing the H2L^d antigen were also recognized, but to a lesser extent. These data indicate that all three novel class I products function as targets of the CTL generated against the tumor. In addition, residual reactivity can be detected against the 28^- tumor variant lacking these novel antigens (reference 9, Fig. 5a). This residual reactivity probably reflects the recognition of antigenic molecules distinct from the novel class I products on this tumor. Wortzel et al. (28) have shown that 1591 expresses at least four genetically distinguishable sets of novel tumor antigens, termed A, B, C, and D, as defined by cloned CTL. Three of these antigens, B, C, and D, remain on the 28^- tumor variant (9), and are therefore not related to the novel class I molecules discussed in this report. However, given that none of the tumor-reactive mAbs block the anti-A CTL (29), it is not yet possible to determine the relationship between any of the novel class I antigens and the A target molecule.

Since some anti-1591 CTL activity was directed towards H-2L^d (27.5 cells) (Fig. 5a); we cultured spleen cells from the 1591-immunized (C3H X H-2^dm2)F1 mice with BALB/c (H-2^d) spleen cells. H-2^dm2 is a meiotic mutant of BALB/c that lacks the H-2L^d antigen. Therefore, this procedure should generate a primary anti-H-2L^d response, as well as amplify any anti-H-2L^d crossreactive component that arose from the priming with 1591 cells. The effector cells generated lysed 1591 tumor cells, L cells expressing the A149 antigen, and H-2L^d (Fig. 5b). L cells expressing the A166 product were also killed, but much less efficiently. In contrast to primed cells challenged in vitro with 1591 cells (Fig. 5a), effector cells challenged in vitro with BALB/c spleen cells did not lyse target cells expressing A216 or the 28^- variant tumor line. This indicates that the A216 antigen does not cross react with H-2L^d at the T cell level. It is likely that the differential recognition of A149 and A166 by the anti-H-2L CTL reflects some of the five amino acid differences that exist between the α1 domains of these molecules.

To further study the relationships among H-2L^d, A149, and A166, we tested bulk-cultured anti-H-2L^d CTL, as well as several anti-H-2L^d CTL clones, against these target cells (Table II). (C3H X dm2)F1 anti-BALB/c (anti-H-2L^d) CTL displayed lytic activity against H-2L^d and A149 transfectants, but not L cells transfected with A166 or A216 (Fig. 6, left). Activity was also noted against the
1591 target cells (Fig. 6, right), but not the 28− variant. Four out of seven anti-H-2Ld clones recognized A149 transfectants, while none lysed A166 transfectants (Table II). Taken together with the data presented in Fig. 5b, these results indicate that A149 extensively crossreacts with H-2Ld at the T cell level, while A166 is much less crossreactive.

To further show that the A149 molecule, either expressed on the transfected L cells or the 1591 tumor, crossreacts with allospecific anti-H-2Ld CTL, we tested the ability of A149-expressing cells to block the cytolytic activity of an anti-H-2Ld CTL clone, L13D.17. This clone lysed H-2Ld (27.5) and A149 transfectants, as well as 1591 cells (Fig. 7, a−c). The lysis of any of these three targets could be blocked by adding an excess of 27.5, A149, or 1591 inhibitor cells. No inhibition occurred when Ltk− or the 28− variant inhibitor cells were added, as expected.

Thus, the above data show that the novel H-2L-like antigens expressed on the tumor can serve as targets for alloreactive anti-H-2Ld CTL. In addition, we wished to determine whether these antigens could also function as restriction elements for antigen-specific CTL. Therefore, we analyzed the reactivity of H-2Ld-restricted anti-vesicular stomatitis virus (VSV)1 CTL with VSV-infected L cells expressing the class I molecules from the tumor. The H-2L-like A149 antigen can, in fact, function as a restriction element for these H-2Ld-restricted CTL (Fig. 8, a−c). No lytic activity against A216 or A166 transfectants was shown (Fig. 8c). Thus the hierarchy of recognition of the tumor antigens by the bulk and cloned anti-H-2Ld alloreactive CTL was comparable to that of the H-2Ld-restricted CTL, suggesting perhaps that the same class I structures recognized by allospecific CTL are involved in the function of class I restriction elements.

1 Abbreviation used in this paper: VSV, vesicular stomatitis virus.
FIGURE 7. Percent inhibition of lysis mediated by the anti-H-2L\(^d\) CTL clone L13.D17 in the presence of unlabeled competitor cells. L13.D17 was tested against \(^{51}\text{Cr}\)-labeled L cells transfected with H-2L\(^d\) (A), A149 (B), or 1591 cells (C). Unlabeled inhibitor cells are L cells transfected with H-2L\(^d\) (\(\square\)), A149 (\(\bigtriangleup\)), thymidine kinase (\(\triangle\)), or 1591 tumor cells (\(\triangleleft\)), or the 28' tumor variant (\(\triangleleft\)). Specific release in the absence of inhibitor cells against H-2L\(^d\) L cells (E/T, 10:1), A149 L cells (E/T, 10:1), and 1591 (E/T, 40:1) was 49, 33, 17, respectively.

FIGURE 8. Anti-VSV CTL activity against 1591 class I antigens. Spleen cells from BDF\(_1\) mice that were primed with VSV were challenged in vitro with VSV-infected spleen cells. CTL that were generated were tested against VSV-infected L cells transfected with H-2L\(^d\) (\(\square\)), A149 (\(\bigtriangleup\)), A166 (\(\triangle\)), A216 (\(\triangleleft\)), or tk (\(\Delta\)). Data from three panels represent three separate experiments.

Discussion

In addition to the normal complement of H-2K\(^k\) and H-2D\(^k\), the UV-induced C3H (H-2\(^k\)) fibrosarcoma, 1591, expresses at least three novel class I antigens.
Previous characterization of 1591 and 1591 tumor variants suggests that these novel class I antigens might function as the primary targets of a CTL response in normal histocompatible mice (9, 28). Since the loss of these products correlates with the ability of certain variants of this normally nonmetastatic tumor to escape immune rejection and progress to metastasis in immunocompetent hosts (9, 11), it is clear that they play an essential role in limiting tumor growth in the transplant situation. It is not obvious, however, what effect expression of these products exerted on the progression of the tumor in its original UV-irradiated host. While UV irradiation appears to induce a specific suppression of the ability of normal mice to mount an immune response against UV-induced tumors (5, 28), the immune elimination of other non-UV-induced tumors remains relatively unimpaired (30, 31). Most UV-tumors express unique tumor specific-antigens (9, 31), but the biochemical nature of these antigens and their relationship, if any, to UV-specific suppression remain obscure. The 1591 fibrosarcoma is the first UV-induced tumor for which a molecular description of any tumor antigens is available.

Before it is possible to define the specific immunobiological interactions that the novel class I antigens might mediate when expressed on the developing tumor, we believe that it is necessary to define the functional properties of these molecules in the context of a competent immune system. It is also essential to define the molecular origin of these antigens to determine whether their expression is, for example, a general response to the UV treatment.

To this end we have identified (12) the genes encoding the three novel class I antigens expressed on the 1591 tumor, determined the DNA sequences of the genes and the amino acid sequences of their predicted products, and characterized the functional recognition of these molecules on the 1591 tumor and on L cells transfected with the individual novel tumor genes. The three novel class I genes, A149, A166, and A216, encode polypeptides that are highly homologous to, yet distinct from, previously characterized class I antigens. Since the 1591 tumor does indeed appear to be derived from C3H (9, 12), and the novel genes account for several of the polymorphic restriction fragments observed in genomic Southern blots comparing the class I genes of 1591 with those of normal C3H tissue (12), these unique genes may have been generated by multiple recombination events among the endogenous class I genes of C3H.

In support of this hypothesis, the A216 antigen is extremely homologous to the AKR H-2K\(^k\) antigen throughout its C-terminal domain. The 5'-flanking information encoded on the \(\lambda\) clone containing this gene suggests strongly that the 5' coding information of this gene is derived from the equivalent of one of the cluster 8 genes defined from the BALB/c cosmid library (reference 32 and S. Watts, University of California, Berkeley, unpublished results). The A216 \(\lambda\) clone not only contains a fragment of a second class I gene in a head-to-head orientation, but hybridizes to a low copy probe specific for this cluster (31). Furthermore, this clone contains a 7.7-kb polymorphic Hind III fragment defined as representative of C3H (31), consistent with the C3H origin of this gene. Although the \(H-2K^k\) gene may serve as a 3' parent, the complete description of the events by which A216 was generated awaits further DNA sequence comparison with the appropriate C3H loci.
One of the most intriguing features of this tumor is that 1591 expresses two distinct, highly homologous, and immunologically crossreactive class I antigens. A149 and A166 are >98% homologous to each other and to the H-2L^d antigen from BALB/c. The genes encoding these three antigens are nearly identical for 2,000 bp containing the exons encoding the third external domain, α3, the transmembrane domain, and the cytoplasmic domain. Unique sequences within the 5' ends of the genes account for the discrete functional differences observed between their products. Since a polymorphic Pst I fragment not found in C3H is contained within this region of near identity, it is possible that A149 and A166 were derived by duplication of a recombinant precursor gene, followed by additional recombination events to generate the discrete differences between the two tumor genes. Alternatively, the polymorphic 1.0-kb Pst I fragments found in both A149 and A166 might have been generated independently by comparable recombination events involving two highly conserved parental genes. Additional polymorphic restriction fragments map to the 5' end of the gene, suggesting that multiple recombination events were required to generate each of these novel genes (data not shown). Identification and DNA sequence analysis of all of the class I genes involved in these complex recombination events will probably be necessary to define the precise recombinational pathway by which these novel genes were generated.

The extensive sequence homologies observed among A149, A166, and H-2L^d are also shared with the 3' coding region of the H-2D^b gene. Such unprecedented sequence conservation between class I genes from three different haplotypes is remarkable, and argues for an allelic relationship between these three sets of genes (33). In support of this hypothesis, a 5' flanking probe derived from A149 crosshybridizes to H-2L^d, and detects several class I genes from a normal C3H library, which map to the D end of the H-2 (e. g., H-2D^k). Thus, although C3H is not believed to express H-2L locus information, conserved H-2L coding sequences may exist in an unexpressed configuration within the appropriate region of the MHC.

The extreme structural conservation observed among A149, A166, and H-2L^d is reflected by significant functional homology. The three antigens share both public and private class I antigenic determinants, and most of the antigenic differences that have been observed among these molecules appear to correlate with discrete sequence substitutions. Previously, studies involving the serological characterization of exon-shuffled class I gene products have succeeded in localizing the structures recognized by several different allospecific mAb to individual protein domains (21, 34–37). Unfortunately, attempts to correlate such serological determinants with specific sets of amino acids have been complicated by the comparison of class I products that differ at multiple amino acid residues (38). However, given the ability of antibodies directed against discrete peptides to recognize the native conformation of a protein (39), it is essential to determine whether specific alloantibodies against H-2 react with conformational determinants, or whether it is possible to identify specific coding sequences that can function as independent epitopes.

While both A149 and A166 molecules are closely related to H-2L^d, both by sequence and serological criteria, only the former molecule crossreacts exten-
sively with H-2L<sup>d</sup> at the T cell level. Thus, anti-1591 CTL react against H-2L<sup>d</sup>, A149, A166, and A216, while anti-H-2L<sup>d</sup> CTL crossreact primarily with A149. However, a distinct population of A166 crossreactive CTL could be shown by challenging the spleen cells from tumor immune animals in vitro with H-2L<sup>d</sup>. A149 and A166 differ from H-2L<sup>d</sup> at 6 and 8 amino acid residues, respectively, in the α2 domain. Four of the changes are shared by both A149 and A166, and the additional unique changes found in A166 are no more different, with respect to charge or side-chain bulk, than those seen in A149. In contrast, A166 has 5 amino acid changes in the α1 domain while A149 has none. Thus, these results could indicate that the vast majority of anti-H-2L<sup>d</sup> CTL are controlled by determinants mapping to the α1 domain. This is surprising, however, in light of previous results that mapped some H-2L<sup>d</sup> CTL determinants to the α2 domain. Specifically, Murre et al. (37) have observed that a domain shuffled molecule that contained the α1 of H-2D<sup>d</sup> and the α2 of H-2L<sup>d</sup> retained anti-H-2L<sup>d</sup> CTL determinants. We (J. Forman, unpublished data) have noted that anti-H-2L<sup>d</sup> CTL also readily recognize the H-2<sup>dm1</sup> molecule, which has H-2L<sup>d</sup> sequences only in the carboxy-terminal end of the α2 domain, but H-2D<sup>d</sup> sequences in the α1 and the proximal half of the α2. Perhaps these observations can be reconciled with the data discussed in this report by postulating that specific intramolecular interactions with α1 structures are required to impart a recognizable conformation on α2-encoded determinants. Amino acid substitutions found in the α1 of A166 might somehow interfere with these conformational interactions. Such interactions might not be obvious from the analysis of the shuffled or dm1 molecules described above if the H-2D<sup>d</sup> α1 domain was itself compatible with the α2 of L<sup>d</sup>. This hypothesis can be tested by generating mutant A166 molecules in vitro, in which A166 residues are replaced with H-2L<sup>d</sup> and D<sup>d</sup> sequences.

Conformational interactions between class I domains have been implicated in previous studies involving the H-2K<sup>b</sup> series of class I mutants (40, 41) where one to three amino acid changes in either the α1 or α2 domains usually result in the loss of most of the CTL epitopes. Further evidence for conformational interactions and the requirement for domain compatibility comes from studies involving the shuffling of exons that encode the α1 and α2 domains of class I molecules (34–36). In these studies it was found that virtually all of the CTL epitopes characteristic of the native molecules are absent from the hybrid molecules.

Detailed study of the structural bases of these conformational interactions might be uniquely accessible in the 1591/H-2L<sup>d</sup> system for several reasons: (a) the structural differences that exist between A149, A166, and H-2L<sup>d</sup> are quite discrete and can be easily manipulated by oligonucleotide directed mutagenesis; (b) all three antigens seem able to evoke a CTL response; (c) A149 and A166 each crossreact to some extent with H-2L<sup>d</sup> with respect to both CTL and serological reagents; (d) discrete changes in crossreactivity will probably be observed after in vitro mutagenesis; and (e) H-2L<sup>d</sup> has already been extensively characterized as a T cell target (17, 18, 35).

The crossreactivity of A149 with H-2L<sup>d</sup> was also noted with regard to antigen-specific CTL. Thus, BDF<sub>1</sub> anti-VSV CTL, restricted to H-2L<sup>d</sup>, also recognize VSV antigens in association with A149. These data indicate that the tumor not only expresses a molecule that can function in the restriction of virus-specific
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CTL, but also that the functional homology of A149 to H-2Ld involves determinants recognized by both allospecific and H-2-restricted CTL.

Taken together, our present results indicate that all three novel class I antigens expressed on 1591 can function as potential target structures for the immune destruction of the transplanted tumor in histocompatible hosts. Of course, the immunogenicity of any one of these molecules could depend upon its relative level of expression on the surface of the tumor cell, as well as the immunological status and specific MHC background of the host. It is also difficult to predict the precise effects of the expression of the two highly homologous, but distinct, H-2L-like antigens. Clearly, this tumor could progress in the autochthonous host despite its extreme immunogenicity, and it is quite possible that the expression of these novel antigens was somehow related to the UV-induced immune suppression operative during tumor progression. Further immunobiological studies involving tumor variants and class I transfectants transplanted into UV-irradiated and normal hosts may elucidate the precise effect each of these molecules might mediate during tumor progression.

Summary

The UV-induced, C3H fibrosarcoma, 1591, expresses at least three unique MHC class I antigens not found on normal C3H tissue. Here we report the complete DNA sequence of the three novel class I genes encoding these molecules, and describe in detail the recognition of the individual products by tumor-reactive and allospecific CTL. Remarkably, although C3H does not appear to express H-2L locus information, this C3H tumor expresses two distinct antigens, termed A149 and A166, which are extremely homologous to each other and to the H-2Ld antigen from BALB/c. The gene encoding the third novel class I antigen from 1591, A216, is quite homologous to H-2Kd throughout its 3' end. Since all three of these genes account for polymorphic restriction fragments not found in C3H, it is likely that they were derived by recombination from the endogenous class I genes of C3H. The DNA sequence homology of A149, A166, and H-2Ld is especially significant given the functional conservation observed between the products of these genes. Limited sequence substitutions appear to correlate with some of the discrete serological differences observed between these molecules. In addition, both A149 and A166 crossreact, but to differing extents, with H-2Ld at the level of T cell recognition. Our results are consistent with the view that CTL recognize complex conformational determinants on class I molecules, but extend previous observations by comparing a set of antigens with discrete and overlapping structural and functional differences.

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