NEW FAMILY OF EXON-SHUFFLED RECOMBINANT GENES REVEALS EXTENSIVE INTERDOMAIN INTERACTIONS IN CLASS I HISTOCOMPATIBILITY ANTIGENS AND IDENTIFIES RESIDUES INVOLVED

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MHC-encoded class I antigens are found on the surface of the majority of nucleated mammalian cells and play a central role in the immunosurveillance process whereby virus-infected or malignant cells are destroyed by cytotoxic T lymphocytes (CTLs). Class I antigens are highly polymorphic and consist of a polypeptide chain of ~46 kD organized into three extracellular domains of ~92 residues each (termed N, C1 and C2, numbering from the NH_3 terminus), a transmembrane region, and an intracellular domain (1). This chain is associated noncovalently with the 12 kD polypeptide β_2-microglobulin (β_2m)[1] through its C2 domain. Close structural homology between immunoglobulins and the C2 domain of class I antigens, β_2m, and the membrane-proximal domains of both chains of the class II MHC antigens has been inferred from the well-established sequence homologies between these members of the immunoglobulin superfamily (2, 3), and in the case of β_2m, has been shown crystallographically (4). Until recently, however, little was known about the tertiary structure of the N and C1 domains of class I antigens, which manifest the majority of the sequence polymorphism and are much less homologous to immunoglobulin than is the C2 domain. Although the C1 domain contains a disulfide bond enclosing a loop of 63 residues, the position of the two cysteines are not homologous to their counterparts in immunoglobulins.

The two membrane-distal domains, N and C1, are the most important for the immunological function of the class I antigens. The sites within class I molecules responsible for recognition by both monoclonal antibodies (mAbs) and CTLs have been partially localized and shown to predominantly reside in these two domains (5–12). Much of the localization of mAb and CTL epitopes has used hybrid class I molecules produced by the expression in mouse L cells of recombinant genes in which the exons encoding the individual external domains have been exchanged (5–12). In this work, a new family of hybrid genes was generated

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in which exons encoding the N, C1, and C2 domains were exchanged between the cloned H-2D^d (5) and H-2D^p (13) genes to produce four of the six possible recombinant permutations. Studies with these new recombinant gene products allowed the analysis in some detail of the conformational determinants created by the interaction between the N and C1 domains of class I antigens. The specificities of a large panel of anti-H-2D^d mAbs, the epitopes of which had been assigned to one or both of the distal domains on the basis of experiments with H-2D^d/H-2L^d recombinants (12) and a number of anti-H-2D^p mAbs were examined using the H-2D^d/H-2D^p recombinants. This analysis revealed that a number of epitopes recognized by the anti-H-2D^d mAbs, previously localized to the N domain, were modified by substitutions in the C1 domain. By comparing the primary structures of the C1 domains of the D^d, L^d, and D^p molecules, a small number of candidate residues in the C1 domain responsible for this effect were identified. A high proportion of the anti H-2D^p mAbs also recognized epitopes involving both domains.

The high frequency with which CTLs and mAbs recognize complex determinants dependent upon both N and C1 domains observed here and in other studies (7–9), along with the many distinct patterns of crossreactivity of the mAbs used here with other class I molecules, suggests an extensive area of interaction between these two domains which is of central importance to the function of class I molecules. This idea is supported by the recent findings on the crystallographic structure of a human class I antigen (Samraoui, B., P. Bjorkman, and D. Wiley, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA, personal communication).

Materials and Methods

Preparation of Recombinant Genes.   Plasmid pD^d-1 (5) was digested to completion with Xba I giving a 1.8 kb fragment containing exons 1, 2, and 3, and a 12 kb fragment containing exons 4–8 of the H-2D^d gene plus the pBR322 vector; these fragments were purified from a 1% agarose gel by electroelution followed by DEAE column chromatography. The 1.8 kb fragment was subcloned into the Xba I site of pUC13 (14), producing pD^d-11.

The 1.8 kb and 2.5 kb fragments resulting from complete digestion of clone λ12a, which encodes the H-2D^p gene (13, 15), with Xba I and Bam HI, respectively, were purified and subcloned into the Xba I site of pUC13 and the Bam HI site of pUC9 (14) producing pD^p-11, containing exons 1, 2, and 3, and pD^p-21, containing exons 4–8 of the H-2D^p gene.

Clones pD^d-11 and pD^p-11 were now treated similarly: complete digestion with Sma I produced a 3.5 kb fragment containing exon 3 and the pUC13 vector, which was purified as above; partial Sma I digestion gave fragments of 4.5 kb, 4.2 kb, 3.9 kb, 3.5 kb, 1.0 kb, 0.6 kb, and 0.3 kb; the 1.0 kb band contains exons 1 and 2 and was purified as above. The 1.0 kb Sma I fragment from pD^p-11 was now ligated with the 3.5 kb Sma I fragment from pD^d-11 and vice versa, producing clone a and clone b, respectively.

Exons 1, 2, and 3 were then removed from clones a and b on the 1.8 kb Xba I fragments and ligated into the 12 kb fragment produced by Xba I digestion of pD^d-1. The other recombinant clones were produced by cutting pD^p-21 with Hind III, filling in the 5' protrusions with Klenow fragment and blunt-end-ligating to the 1.8 kb Xba I fragment, which had been similarly filled in with Klenow fragment, from clone a, clone b, pD^d-1 and pD^p-11 (see Fig. 1).

Production of Transformed Cell Lines Expressing Recombinant H-2 Genes.   The recombinant H-2D^d/H-2D^p genes were transfected into mouse L cells (DAP-3) and transformed
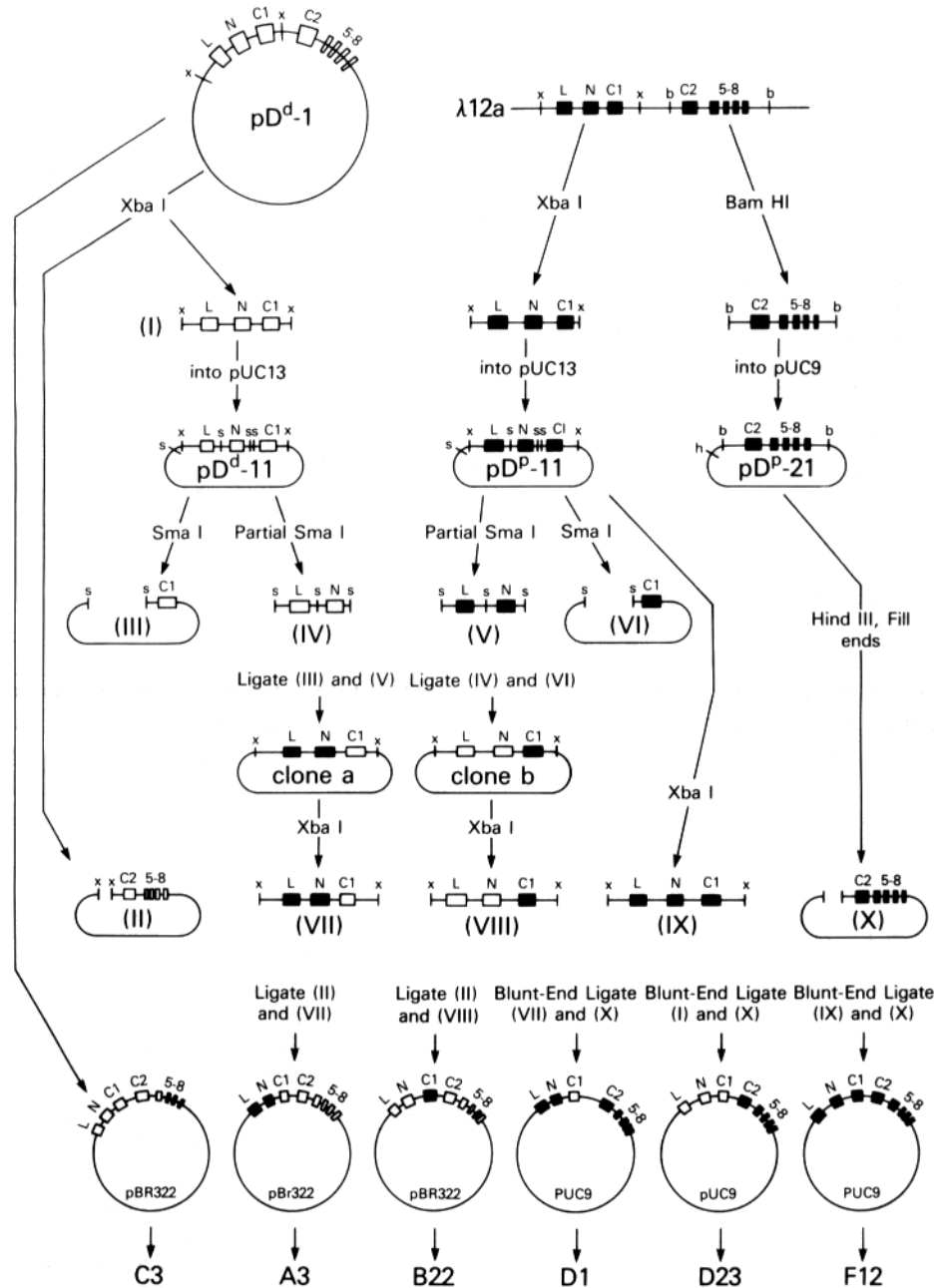
FIGURE 1. Recombination of exons between H-2D^d and H-2D^p genes. Exons are indicated by boxes, open for those encoded by the D^d gene and closed for those derived from the D^p gene. Restriction sites: x, Xho I; b, Bam HI; s, Sma I; h, Hind III.

clones were selected exactly as described previously (16). Untransformed DAP cells for use as negative controls were maintained in DMEM (Flow Laboratories, McLean, VA) supplemented with 10% FCS, 2 mM glutamine, and 10 μg/ml gentamycin under an

atmosphere of 5% CO_2. Transformed cells were grown in the same medium supplemented with HAT.

Monoclonal Antibodies. The mAbs used in this work, their specificities, and their patterns of crossreactivity with other haplotypes has been described previously (12, 17). Antibodies 135 and 218 were supplied by Dr. Kim Hasencrug, McLaughlin Research Institute, Great Falls, MT.

Antibody Binding to Transformed Cells. Antibody binding to the cells containing the recombinant H-2 genes was measured in an indirect radioimmunoassay, essentially as described previously (12). Cells were harvested by trypsinization and plated out in 96-well microtiter plates (Costar, Cambridge, MA) at densities of 0.8–1.7 × 10^6 cells/ml in volumes of 100 µl. After growing overnight, the cell layers were washed three times with PBS containing 10% FCS (PBS/FCS), then incubated with 30 µl of mAb in tissue culture supernatant, also containing 10% FCS, for 1 h at 4°C. This amount of antibody was sufficient to ensure saturation of antigens. After three more washes with PBS/FCS, bound antibody was detected with 50 µl of ^{125}I-conjugated sheep anti–mouse immunoglobulin (Amersham Corp., Arlington Heights, IL) at dilutions of 1:100 to 1:160 for 1 h at 4°C. The unbound antibody was removed by three washes with PBS/FCS, after which cells were lysed in 100 µl of 1% NP-40 (BDH Chemicals Inc., Poole, United Kingdom). This solution was harvested onto filter plugs using a titertek cell harvester (Flow Laboratories) and ^{125}I-labelled material was detected in a Beckman 5500 gamma counter. Alternatively, for anti-H-2D^p mAbs, ^{125}I–protein A was substituted for anti–mouse IgG and plates were cut and counted directly. For IgM antibodies, affinity-purified rabbit anti–mouse µ chain was used as an intermediate. All data points were determined in triplicate.

Results

Binding of Anti-H-2D^d mAbs to Recombinant Class I Gene Products. The reactivity of each of the panel of anti-H-2D^d mAbs with transformed cell lines expressing the exon-shuffled class I proteins, along with the domain assignment of each epitope from these results and from previous experiments with D^d/L^d recombinants (12) is shown in Table I. The results with the test antibodies and with the controls 34.5.8 and 34.2.12 indicate that the level of expression of the transfected genes in the cell lines A3 (pdd), B22 (dpd), and D1 (pdp) is somewhat lower than in the others. Maximal binding of antibodies to these three lines appears to be 70–80% of that to C3, D23, and F12. Reactivity of an antibody with a particular cell line is considered to be significantly reduced if it is 20% or less of the binding to C3, which expresses the wild type D^d molecule.

The domain assignments from the new D^d/D^p recombinants generally confirm those made previously (12), or resolve ambiguities in them. Antibodies T0.101, T0.102, and T0.103, previously known to crossreact with cells from mice of the H-2^p haplotype, do not bind to the D^p molecule expressed on cell line F12, which suggests that they are crossreactive with the K^p molecule. Antibodies T0.112, T0.113, T0.114, T0.117, T0.121, and T0.123, which were previously shown to recognize epitopes either within C1 or dependent upon both N and C1, on the basis of these new recombinants, have epitopes wholly within C1. Antibody T0.138, on the contrary, previously indistinguishable from these mAbs, recognizes an epitope dependent on both domains.

The results for some of the anti-H-2D^d antibodies with the D^d/D^p recombinants, however, yield new information concerning their specificities not previously revealed by the experiments with the D^d/L^d recombinants. 12 mAbs, namely T0.108, T0.109, T0.116, T0.126, T0.127, T0.132, T0.134, T0.135, T0.139, T0.141, T0.143, and T0.145, whose epitopes were previously mapped

to the N domain of D^d, are now demonstrated to show greatly reduced (<20%) binding to B22 cells expressing a construct where the N domain of D^d is placed adjacent to the C1 domain of D^p. A further six mAbs, T0.104, T0.115, T0.122, T0.128, T0.142, and T0.144, show binding to B22 cells at 20–50% of the control. All of these antibodies display full crossreactivity between the wild type D^d molecule and a construct where the N domain of D^d is placed adjacent to the C1 domain of L^d (12). This result implicates those positions in the C1 domain common to D^d and L^d but differing in D^p as being involved in their epitopes (see Discussion).

Antibodies T0.110 and T0.111 display curious patterns of reactivity. The binding of T0.110 to cell lines A3 and D1 (N-D^p, C1-D^d) is very low, while it binds at 50% of the control level to F12 (N-D^p, C1-D^p). Thus, substituting the N domain abolishes binding, which can be partially restored by further substituting the C1 domain. The antibody recognizes both wild type molecules better than any recombinant where the N or C1 domain has been substituted. T0.111, on the contrary, reacts with all recombinants (N-D^d, C1-D^p or N-D^p, C1-D^d), but not with the wild type D^p on F12 cells.

Binding of Anti-H-2D^p mAbs to Recombinant Class I Gene Products. Table II shows the reactivity of each of the anti-H-2D^p mAbs with the D^p/D^d recombinants and the assignment of their epitopes to particular domains based on these data. Two antibodies, 7-30.6 and 11-20.3, appear to react with epitopes dependent solely upon the N domain of D^p. In contrast, five antibodies, 5-7.1, 218, 7-30.2, 7-16.10, and 135, require both N and C1 domains to be of D^p origin in order to bind. Antibody 6-27.5 has a pattern of reactivity that is hard to explain in terms of domain requirements. It shows broad crossreactivity with other class I molecules (reference 17 and Table IV), which suggests that it recognizes a relatively nonpolymorphic section of the molecule.

mAb Reactivity with Other Class I Molecules. Comparing the crossreactivity profiles (12) of the 15 mAbs that are specific for complex epitopes involving both N and C1 domains of H-2D^d (Table III A) shows that they have eight distinct patterns of crossreactivity. As seven of these antibodies have private specificities and therefore cannot be distinguished by such experiments, it is very likely that more than eight distinct epitopes actually exist. Similarly, the five anti-H-2D^p mAbs specific for N/C1 complex–determinants display five distinct crossreactivity patterns (Table III B). Thus the anti-D^p mAbs also recognize multiple distinct epitopes in the region of interaction of the N and C1 domains of the class I molecule.

Discussion

Previous attempts to localize the epitopes recognized by alloantibodies or CTLs on class I molecules have mostly addressed the question of which domain or domains contain these epitopes (5–12). The results suggest that the great majority of CTLs recognize structures dependent on both the N and C1 domains, and are unaffected by the C2 domain. A high proportion of alloantibodies recognize distinct determinants in the N and/or C1 domains, although some mAbs have been characterized that see epitopes within the C2 domain (5, 7).

TABLE I

Binding of Anti-H-2D^d mAbs to Cells Expressing Recombinant Class I Genes

mAb	Binding (cpm)* by C3 ddd	Percent of cpm bound by C3‡						Domain assignment§	
		A3 pdd	B22^l dpd	D23 ddp	D1 pdp	F12 ppp	DAP	L^d/D^d	D^p/D^d
T0.101	4,264	0	1	89	0	0	0	—	N,C1
T0.102	5,375	64	39	102	65	95	0	—	—
T0.103	4,814	64	73	87	68	102	0	N	—
T0.104	3,490	1	23	79	0	1	0	N	N(C1)
T0.105	1,251	2	11	104	2	7	1	—	N,C1
T0.106	6,523	74	0	91	73	1	0	—	C1
T0.107	4,383	1	66	93	0	0	0	N	N
T0.108	1,195	1	12	106	3	8	2	N	N,C1
T0.109	1,338	3	13	105	3	7	1	N	N,C1
T0.110	1,097	5	27	110	8	47	2	N	—
T0.111	3,865	76	57	98	68	0	0	N	—
T0.112	3,876	77	1	112	73	0	0	C1/N, C1	C1
T0.113	3,726	74	1	119	72	0	0	C1/N, C1	C1
T0.114	3,588	83	1	104	76	1	1	C1/N, C1	C1
T0.115	5,212	0	38	98	0	1	1	N	N(C1)
T0.116	753	3	3	107	2	1	4	N	N,C1
T0.117	5,505	65	0	113	64	0	1	C1/N, C1	C1
T0.119	5,441	0	83	79	1	1	1	N	N
T0.120	4,611	1	51	71	0	0	0	N	N
T0.121	5,195	86	1	112	82	0	0	C1/N, C1	C1
T0.122	861	1	48	104	1	6	2	N	N(C1)
T0.123	5,764	67	0	94	76	0	0	C1/N, C1	C1
T0.124	4,718	1	74	91	0	1	1	N	N
T0.126	861	3	2	98	1	3	2	N	N,C1
T0.127	871	3	16	78	5	3	1	N	N,C1
T0.128	3,615	0	24	93	1	1	1	N	N(C1)
T0.129	5,764	0	82	89	0	0	0	N	N
T0.130	3,731	7	71	101	7	0	1	N	N
T0.131	3,266	0	70	81	1	0	1	N	N
T0.132	3,124	0	1	89	0	1	1	N	N,C1
T0.133	3,349	1	70	80	1	1	1	N	N
T0.134	4,992	1	0	81	0	1	1	N	N,C1
T0.135	2,301	1	8	90	2	1	1	N	N,C1
T0.136	5,024	0	74	87	0	0	1	N	N
T0.137	5,961	0	67	92	1	1	0	N	N
T0.138	4,101	1	3	92	0	0	0	C1/N, C1	N,C1
T0.139	621	2	14	51	0	2	4	N	N,C1
T0.141	5,142	0	0	82	1	0	1	N	N,C1
T0.142	427	2	39	59	8	7	2	N	N(C1)
T0.143	329	4	12	68	0	1	0	N	N,C1
T0.144	3,087	1	32	92	0	0	0	N	N(C1)
T0.145	4,178	0	3	83	0	0	0	N	N,C1
34.5.8	5,713	77	0	114	73	0	0	C1	C1
34.2.12	5,035	86	65	0	0	0	0	C2	C2
16.1.2	4,509	121	110	97	106	97	87	D^k/K^k	D^k/K^k
Medium¶	1	0	0	1	0	0	1		

TABLE II

Binding of Anti-H-2D^p mAbs to Cells Expressing Recombinant Class I Genes

mAb	Binding (cpm) by F12 ppp	Percent of cpm bound by F12					Domain assignment
		A3 pdd	B22 dpd	C3 ddd	D1 pdp	D23 ddp	
5-7.1	33,132	7	5	7	6	6	N,C1
218	20,274	12	10	12	10	13	N,C1
6-27.5	28,183	47	27	26	38	66	—
7-30.2	19,152	17	16	13	13	9	N,C1
7-30.6	23,586	93	25	27	71	18	N
11-20.3	9,629	158	16	36	121	17	N
7-16.10	28,051	2	12	2	2	1	N,C1
135	23,543	0	5	3	2	1	N,C1

Domain compositions of expressed class I antigens and domain assignments of epitopes are as described for Table I. Binding of an irrelevant, K^p-specific antibody, 7-16.4, was always <10% of the binding of a K^k-specific positive control to each transfected cell line.

This implies a certain level of dichotomy between the epitopes on class I antigens recognized by mAbs and CTLs.

This study further investigated the specificities of a panel of anti-H-2D^d mAbs previously characterized according to their reactivities with D^d/L^d recombinants, now using D^d/D^p recombinants. Comparison of the data from the experiments with the two sets of recombinants allows the nature of the mAb epitopes to be analyzed in more detail than is possible from either set alone. The particular pattern of polymorphism manifest in class I antigens, where many positions are variable but a relatively low number of amino acid variants is seen at any one position, means that any two class I antigens will share the same residues at a number of polymorphic positions. Analysis of mAb (or CTL) specificities using a single set of exon-shuffled products is thus limited by the fact that the exchanged exons may be identical in positions recognized by certain mAbs (or CTLs). This problem can be minimized by using recombinants made from more than two genes, as is clearly demonstrated in this work. The limited number of positions at which any pair of class I molecules differ is now an advantage as it allows an assessment of the contributions of individual amino acids to the antigenic structure of the molecules.

In this work, we have shown that a high proportion of mAbs (at least 20 out of 49) recognizing the N and/or C1 domains of the D^d or D^p molecules are actually specific for epitopes the integrity of which requires both of these two domains. The same phenomenon has been observed in the K^d/K^k system (8). In

* The results represent the mean of triplicate determinations.
‡ The domain compositions of the recombinant genes expressed in cell lines C3, A3, B22, D23, D1, F12, and DAP are indicated in the order: N, C1, C2 (d, D^d; p, D^p).
§ The epitope recognized by each antibody is assigned to one or two domains on the basis either of previous experiments with L^d/D^d recombinants (12) or on the basis of the data presented here: N, N domain; C1, C1 domain; N,C1, requiring residues in both domains; N(C1), N domain with a possible contribution from C1 domain (20–50% of the control binding to B22 cells); C1/N,C1, either C1 domain alone or N and C1 domains together.
¶ Binding to B22 cells was determined in two independent experiments which gave very similar results. Data from one of the two experiments are shown here.
# Binding (cpm) by medium control in the absence of primary antibody is expressed as a percentage of binding by mAb 16.1.2, specific for the H-2^k molecules constitutively expressed by DAP cells.

Table III

Crossreactivity Profiles of N,C1-specific Antibodies

Molecule	Monoclonal antibody*																				
	01	05	08	09	16	26	27	32	34	35	38	39	41	43	45	(04	15	22	28	42	44)
A. Anti-H-2D^d																					
D^d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L^d	+	+																			
N-D^d,C1-L^d	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
N-D^d,C1-D^p																					
D^p																					
D^b	+	+																			
K^d	+																+	+			
K^b	+	+		+														+			
K^k																					
K^p	+																				
H-2^a	+	+	+	+	+			+									+	+	+		
H-2^q	+	+	+	+		+	+										+	+	+		
H-2^r			+	+																	
H-2^f	+																				
B. Anti-H-2D^p		5-7.1		7-30.2		7-16.10			218		135										
D^d																					
L^d																					
D^p		+		+		+			+		+										
D^b																					
K^d		+				+			+												
K^b																					
K^p									+												
H-2^a																					
H-2^q																					
H-2^r		+				+			+												
H-2^f		+							+		+										

* The mAbs are indicated in the abbreviated form: T0.101 as 01, etc. A plus sign shows crossreactivity measured by indirect radioimmunoassay for the N-D^d, C1-L^d, and N-D^d, C1-D^p recombinants as described in Materials and Methods, or by flow cytofluorimetry for the other antigens (12). Those antibodies showing 20–50% binding to B22 cells (dpd) are enclosed in parentheses.

addition, antibodies, both polyclonal and monoclonal, raised against a class I molecule containing the N domain of D^p and the C1 and other domains of D^d recognize epitopes not present on either parental molecule. These neodeterminants display a degree of immunodominance in the humoral response to the hybrid molecule (Kanda, LuPan, Takahashi, Appella, and Frelinger, *Immunogenetics*, in press). These observations suggest that the interaction of the two membrane-distal domains is of critical importance to the antigenic structure of class I molecules. The analysis of the contacts between the N and C1 domains will thus be of great importance for an understanding of class I function.

By comparing the reactivities of a large number of mAbs with two panels of recombinant class I gene products (D^d/L^d and D^d/D^p), we have been able to begin to analyze the nature of the N/C1 domain interaction. The 15 anti-H-2D^d mAbs and the five anti-H-2D^p mAbs specific for N/C1 complex determinants recognize at least 15 distinct epitopes, as revealed by their patterns of crossreactivity with other H-2 haplotypes (Table III). This implies that the area of interaction between N and C1 involves a significant portion of both domains.

18 of the anti-H-2D^d mAbs that were assigned as N domain–specific on the

basis of their reactivities with H-2D^d/H-2L^d recombinants are now seen to show reduced reactivity with the B22 cell line, which expresses a molecule which derives its N domain from D^d but its C1 domain from D^p. This implicates those amino acid residues in the C1 domain, where D^d and L^d are the same but D^p is different, as playing a key role in the epitopes recognized by these antibodies. Fig. 2 shows that only at positions 5, 7, 17, 73, 83, 84, and 91 are these criteria met. It is also possible that positions 9, 31, 65, and 66, where all three sequences are different, may be involved. The nature of the substitutions at the latter three positions, however, makes their involvement unlikely, as the changes in L^d relative to D^d are at least as nonconservative as the changes in D^p. The involvement of some or all of these C1 residues in the mAb epitopes could be either direct, if they actually formed a complex epitope with residues located in the N domain and adjacent in the tertiary structure, or indirect, if the nature of these residues in the C1 domain were responsible for conformational perturbations of epitopic residues in the N domain. In either case, the data presented here suggest that these residues are located at positions in the structure intimately involved in the interaction between the N and C1 domains.

Crystallographic studies on the human class I antigens HLA.A2 and HLA.A28 are in progress (18). The most recent interpretation of the structure of the A2 molecule at high resolution reveals that what have been known as the N and C1 domains really are not domains in the strict sense, although we will continue to refer to them as such. Instead they fold to form a single "superdomain." The tertiary organization of this superdomain appears to be quite unlike that of the classic immunoglobulin fold, which is displayed by the C2 domain and β_2m (Samraoui, Bjorkman, and Wiley, personal communication).

This information confirms the conclusion suggested by the results presented in this paper; that there is a strong interaction between the N and C1 domains, extending over a large area. It also suggests that residues in the C1 domain will influence epitopic residues in the N domain by both direct and indirect means. Recent studies (19, 20) have shown that the region of a protein antigen that makes contact with the binding site of an antibody, i.e., constitutes an epitope,
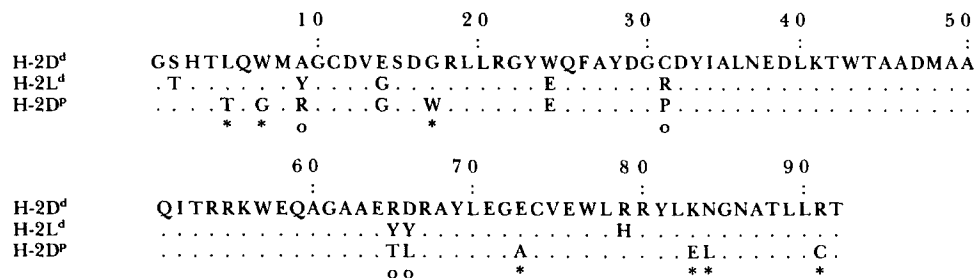
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                   1 0         2 0         3 0         4 0         5 0
                    :           :           :           :           :
H-2Dd    GS HTLQWM AGCDVES DG RLLRGY WQF AYDG CDYIALNEDLKTWTAADMAA
H-2Ld    .T......Y....G.............E.......R....................
H-2Dp    ....T.G.R....G..W......E......P....................
              *  *  o           *                   o

                   6 0         7 0         8 0         9 0
                    :           :           :           :
H-2Dd    QITRRKWEQAGAAERDRAYLEGECVEWLRRYLKNGNATLLRT
H-2Ld    ................YY............H............
H-2Dp    ...............TL......A..........EL......C.
                         o o          *          * *       *
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FIGURE 2. Amino acid sequences of C1 domains of H-2D^d, H-2L^d, and H-2D^p. Residues are numbered from the first position in the C1 domain, i.e., beginning at residue 91 when numbered from the NH$_3$ terminus of the complete molecule. A dot indicates identity to the H-2D^d sequence. Positions indicated with an asterisk are those where the D^d and L^d are the same but D^p is different; an open circle indicates the positions where all three sequences are different. References: H-2D^d (21), H-2D^p (22), H-2L^d (23). The latter differs from the L^d sequence in (24); it is given because it is the sequence of the clone from which the L^d/D^d recombinants were generated.

may be up to 30 × 20Å in size. C1 domain residues will clearly be within this distance of N domain residues on the surface of the class 1 antigen if the two domains actually fold into a single unit. They will also be in positions whereby they might exert indirect effects on the conformations of other residues.

The accumulating evidence points to the region of interaction of the N and C1 domains as being of prime importance for the immune recognition of class I molecules by both antibodies and CTLs. The polymorphism of class I genes has presumably been selected by evolution to maximize the potential for the recognition of their products in association with foreign antigens by CTLs. Thus it is probable that mutations causing changes in class I antigens at the sites recognized by CTLs will be most likely to be preserved. We might therefore predict that the more polymorphic regions of the class I sequences be clustered in the three-dimensional structure in the region of the molecule recognized by CTLs, i.e., at the region of interaction between the N,C1 domains. This hypothesis is supported by the large proportion of mAbs that are also seen to recognize N, C1 complex epitopes if suitable domain exchanges are performed, and which tend to be directed towards the more polymorphic regions of the molecule by virtue of their mode of production. The accuracy of these predictions will be revealed when the complete high-resolution crystal structure of one or more class I molecules is available.

Summary

The specificities of an extensive panel of anti-H-2D^d monoclonal antibodies, which had been previously characterized using exon-shuffled H-2D^d/H-2L^d molecules and a number of anti-H-2D^p antibodies, were examined using H-2D^d/H-2D^p recombinants. The use of this new family of recombinant antigens revealed extensive interaction between the membrane-distal (N and C1) domains of class I molecules. 20 out of 48 mAbs recognize complex epitopes formed by the interaction of these two domains. These antibodies exhibit a number of distinct patterns of crossreactivity with other class I proteins, revealing the presence of multiple epitopes within the region of domain interaction. Comparison of the data presented here with those from previous work allowed the identification of a small number of residues in the C1 domain that participate in the generation of complex epitopes involving both the N and C1 domains. The results are discussed in terms of the structural information available for these two domains.

References

1. Coligan, J. E., T. J. Kindt, H. Uehera, J. Martinko, and S. G. Nathenson. 1981. Primary structure of a murine transplantation antigen. *Nature (Lond.).* 291:35.
2. Orr, H. T., D. Lancet, R. J. Robb, J. A. Lopez de Castro, and J. A. Strominger.

1979. The heavy chain of human histocompatibility antigen HLA-B7 contains an immunoglobulin like region. *Nature (Lond.)*. 282:266.

3. Tragardh, L., L. Rask, K. Wiman, J. Fohlman, and P. A. Peterson. 1980. Complete amino acid sequence of pooled papain solubilized HLA-A, -B and -C antigens: Relatedness to immunoglobulins and internal homologies. *Proc. Natl. Acad. Sci. USA.* 77:1129.

4. Becker, J. W., and G. N. Reeke, Jr. 1985. Three dimensional structure of β_2-microglobulin. *Proc. Natl. Acad. Sci. USA.* 82:4225.

5. Evans, G. A., D. H. Margulies, B. Shykind, J. G. Seidman, and K. Ozato. 1982. Exon shuffling: Mapping polymorphic determinants on hybrid mouse transplantation antigens. *Nature (Lond.)*. 300:755.

6. Murre, C., E. Choi, J. Weis, J. G. Seidman, K. Ozato, L. Liu, S. J. Burakoff, and C. S. Reiss. 1984. Dissection of serological and cytolytic T lymphocyte epitopes on murine major histocompatibility antigens by a recombinant H-2 gene separating the first two external domains. *J. Exp. Med.* 160:167.

7. Bluestone, J. A., M. Foo, H. Allen, D. Segal, and R. A. Flavell. 1985. Allospecific cytolytic T lymphocytes recognise conformational determinants on hybrid mouse transplantation antigens. *J. Exp. Med.* 162:268.

8. Arnold, B., U. Horstman, W. Kuon, H-G. Burgert, G. Hämmerling, and S. Kvist. 1985. Alloreactive T-cell clones preferentially recognize conformational determinants on histocompatibility antigens: Analysis with genetically engineered hybrid antigens. *Proc. Natl. Acad. Sci. USA.* 82:7030.

9. Allen, H., D. Wraith, P. Pala, B. Askonas, and R. A. Flavell. 1984. Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites. *Nature (Lond.)*. 309:279.

10. Ozato, K., G. A. Evans, B. Shykind, D. H. Margulies, and J. G. Seidman. 1983. Hybrid H-2 gene products assign domains recognised by alloreactive T-cells. *Proc. Natl. Acad. Sci. USA.* 80:2040.

11. Levy, R. B., K. Ozato, J. C. Richardson, and J. A. Bluestone. 1985. Molecular localization of allogenic and self determinants recognised by bulk and clonal populations of cytotoxic T-cells. *J. Immunol.* 134:677.

12. Ozato, K., H. Takahashi, E. Appella, D. W. Sears, C. Murre, J. G. Seidman, S. Kimura, and N. Tada. 1985. Polymorphism of murine major histocompatibility class I antigen: Assignment of putative allodeterminants to distinct positions of the amino acid sequence within the first external domain of the antigen. *J. Immunol.* 134:1749.

13. Macchi, M. J., J. G. Woodward, E. McLaughlin-Taylor, J. Griffin, L. Hood, and J. A. Frelinger. 1984. Cloning and identification of the H-2D^P gene. *Immunogenetics.* 19:195.

14. Viera, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19:259.

15. McLaughlin-Taylor, E., J. G. Woodward, M. J. Macchi, M. McMillan, and J. A. Frelinger. 1984. Functional characterisation of the H-2D^P gene. *Immunogenetics.* 19:205.

16. Margulies, D. H., G. A. Evans, K. Ozato, R. D. Camerini-Otero, K. Tanaka, E. Appella, and J. G. Seidman. 1983. Expression of H-2D^d and H-2L^d mouse major histocompatibility antigen genes in L cells by DNA-mediated gene transfer. *J. Immunol.* 130:463.

17. Harmon, R., N. Stein, and J. A. Frelinger. 1983. Monoclonal antibodies reactive with H-2 determinants. *Immunogenetics.* 18:541.

18. Bjorkman, P. J., J. L. Strominger, and D. C. Wiley. 1985. Crystallisation and x-ray

diffraction studies on histocompatibility antigens HLA-A2 and HLA-A28 from human cell membranes. *J. Mol. Biol.* 186:205.

19. Amit, A. G., R. A. Mariuzza, S. E. V. Phillips, and R. J. Poljak. 1986. Three-dimensional structure of an antigen-antibody complex at 2.8A resolution. *Science (Wash. DC).* 233:747.

20. de la Paz, P., B. J. Sutton, M. J. Darsley, and A. R. Rees. 1986. Modelling of the combining sites of three anti-lysozyme monoclonal antibodies and of the complex between one of the antibodies and its epitope. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:415.

21. Sher, B. T., R. Nairn, J. E. Coligan, and L. Hood. 1985. DNA sequence of the mouse H-2D^d transplantation antigen gene. *Proc. Natl. Acad. Sci. USA.* 82:1175.

22. Shepart, B. S., H. Takahashi, K. M. Cozad, R. Murray, K. Ozato, E. Appella, and J. A. Frelinger. 1986. The nucleotide sequence and comparative analysis of the H-2D^p class I gene. *J. Immunol.* 136:3489.

23. Evans, G. A., D. H. Margulies, R. D. Camerini-Otero, K. Ozato, and J. G. Seidman. 1982. Structure and expression of a mouse major histocompatibility antigen gene, H-2L^d. *Proc. Natl. Acad. Sci. USA.* 79:1994.

24. Moore, K. W., B. T. Sher, Y. H. Sun, K. A. Eakle, and L. E. Hood. 1982. DNA sequence of a gene encoding a BALB/c mouse L^d transplantation antigen. *Science (Wash. DC).* 215:679.