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Genetics

Structure and evolution of transplantation antigens: Partial amino-acid sequences of H-2K and H-2D alloantigens

(indirect immunoprecipitation/microsequence analysis/sequence homology/evolutionary models)

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ABSTRACT **Techniques for the amino acid sequence analysis of subnanomole quantities of polypeptides have been applied to characterize β_2-microglobulin and transplantation antigens of the mouse isolated from spleen cells by indirect immunoprecipitation. Eleven residues were identified throughout the NH_2-terminal 27 residues of the β_2-microglobulin; all were identical to residues seen at the corresponding positions of β_2-microglobulins from other species. Two K and two D transplantation antigens were examined and the following generalizations emerged from the limited partial amino-acid sequence data: (*1*) the K and D molecules are homologous to one another; (*2*) they do not show amino acid sequence homology with immunoglobulins; (*3*) the two K and two D molecules differ from one another by multiple amino acid substitutions; and (*4*) the K molecules as a class cannot be distinguished from the D molecules as a class. The genetic and evolutionary implications of these observations are discussed.**

The major histocompatibility complex of mammals is a genetic region which encodes a variety of cell surface antigens, all of which seem interrelated with the immune system. Classically, this complex was defined in mice by rapid graft rejection triggered by genetic differences at this region. The existence of inbred and congenic mouse strains (strains genetically identical but for a particular region) has permitted a detailed genetic analysis of this region (1). In the mouse, the major histocompatibility complex, or the H-2 complex, can be divided by recombinational studies into four major regions—K, I, S, and D (1). These regions code for three major classes of gene products: (*1*) the H-2K and H-2D gene products which represent the classical transplantation antigens (2); (2) the I region gene products which are coded by a region into which the immune response genes map (3); and (3) the S region gene products which appear to code for certain complement components found in the serum (4, 5). By recombinational analysis, the H-2 complex has DNA sufficient to code for up to 2000 structural proteins of molecular weight about 20,000, depending on the amount of interspersed regulatory and nonfunctional DNA.

The H-2K and H-2D gene products exhibit extensive genetic polymorphism. Among the standard inbred mouse strains approximately nine alleles of the K and nine alleles of the D loci have been defined by serological techniques (1). When wild mice from different localities are examined, each new breeding unit (deme) appears to have new D and K alleles (6), suggesting a remarkable degree of polymorphism among the world-wide mouse population. The K and D gene products are found on almost all tissues. Their function(s) is unknown.

The H-2D and H-2K cell surface antigens are hydrophobic glycoproteins of molecular weight 45,000 that can be isolated by indirect immunoprecipitation using specific alloantisera (7). These molecules are noncovalently associated with β_2-microglobulin (8), a polypeptide homologous to the constant region domains of immunoglobulin molecules (9). Numerous peptide map differences between the K and D gene products suggest that the multiple serological differences have their basis in multiple amino-acid substitutions (10). The chemical studies as well as the presence of serological cross reactivities (11) are consistent with the supposition that the K and D genes (or clusters of genes) arose by a process of gene duplication from a single ancestral gene (12, 13). Others have suggested that the major histocompatibility complex may have given rise to gene families coding for other cell surface receptors such as the immunoglobulins (14–17).

We have developed microsequencing techniques to analyze subnanomole quantities of cell surface proteins so that some of these hypotheses may be tested directly by amino acid sequence analysis of the H-2 gene products (18–20). In this paper we present the partial NH_2-terminal amino-acid sequences of the mouse β_2-microglobulin as well as those of products of two K and two D alleles. These data allow us to discuss (*1*) homology relationships among the K and D molecules; (2) the lack of homology among the transplantation antigens and immunoglobulins; and (3) the nature of the amino acid sequence diversity seen among the K and D allelic products.

MATERIALS AND METHODS

Antisera and Mice. Specific K and D region antisera D-4, D-23, D-28b, and D-33 directed, respectively, against the D^d, K^k, D^b, and K^b regions*, were obtained from Jackson Laboratories via the Transplantation and Immunology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Information concerning the preparation of these antisera may be obtained from the National Institutes of Health. Mice of strains B10.A (4R) and B10.A (5R), which are genotypically K^kD^b and K^bD^d, respectively, were obtained from our own mouse colony.

Strategy. Spleens were removed from mice and the cells were cultured *in vitro* for 4–6 hr with groups of tritiated amino acids (i.e., either alanine, lysine, and valine or leucine, proline, and tyrosine) (19). The radiolabeled H-2 gene products were isolated by indirect immunoprecipitation with specific alloantisera as previously described (7, 8, 21). The radiolabeled K and D gene products from one mouse spleen (about 100 pmol) were purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis, eluted from gels, concentrated, and sequenced in the presence of carrier ovalbumin (which has a blocked α-amino group) in a Beckman

* The capital letter *K* or *D* refers to the genetic region involved while the lower case superscript refers to the "allelic" form of that region.

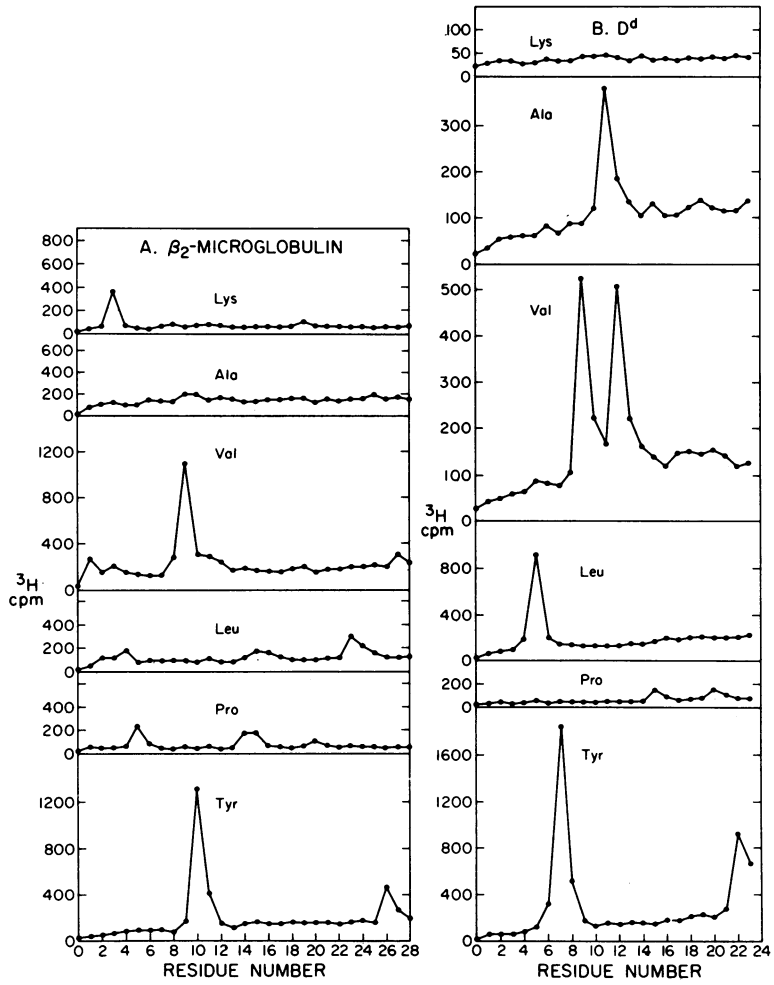

FIG. 1. (A) Amino-acid sequence data from the H-2-associated polypeptide (β_2-microglobulin). (B) Amino-acid sequence data from the D^d gene product. The amount of radioactivity associated with each of the six incorporated amino acids is plotted against residue number (see *text*).

model 890A sequencer as previously described (18–20)†. The tritiated phenylthiohydantoin amino-acid residues were resolved by thin-layer chromatography in the presence of the appropriate unlabeled phenylthiohydantoin amino acids and their radioactivity was measured. Others have independently developed similar techniques for microsequencing (22, 23). Complete details of these procedures will be published in a separate communication (Silver and Hood, in preparation).

RESULTS

Isolation of Two Molecular Components with Alloantisera. Because of the availability of inbred strains of mice that differ solely at restricted portions of the H-2 complex, alloantisera can be prepared that are directed primarily against the K or D gene products. These antisera can be used to isolate K and D gene products by indirect immunoprecipitation (7, 21). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of these immunoprecipitates reveals two major molecular weight components, about 45,000 and 12,000, which are respectively the putative K or D gene products and the putative β_2-microglobulin molecule (see ref. 8).

Amino-Acid Sequence Analysis of the 12,000 Dalton Component. The sequence data obtained from the small polypeptide are illustrated in Fig. 1A. The data display several characteristics typical of conventional automated sequence analysis. There is a gradually rising background due to random hydrolysis of the protein. Sequence residues are characterized by a sharp rise in radioactivity associated with a particular amino acid followed by a more gradual decline. The latter phenomenon, known as "lag," is the result of an incomplete Edman reaction and tends to increase throughout the run. We believe the amino-acid residue present in lowest yield, lysine at position 19, is real in that three separate analyses of the β_2-microglobulin molecule have each given this residue with virtually no background noise in preceding or successive residues. For those residues that appear two or more times (e.g., tyrosine, valine, proline) an average repetitive yield can be calculated. Since each amino acid is incorporated to a different extent, the data for each amino acid must be treated independently from those of the other amino acids. The repetitive yields for these three residues ranged between 89 and 94%, which is similar to the repeti-

† The mouse spleen has 10^8 cells. A spleen cell appears to have approximately 5×10^5 H-2 molecules on its cell surface (37). Accordingly, a single spleen should theoretically yield 5×10^{13} molecules or about 100 pmol of H-2 molecules. If the β_2-microglobulin and H-2 antigens are isolated in a 1:1 ratio, a similar yield of β_2-microglobulin will be obtained. This estimate is high because the yield will not be 100% nor are all the cells in the spleen lymphocytes.

	1	2	3	4	5	6	7	8	9	10
Human	Ile	Gln	Arg	Thr	Pro	Lys	Ile	Gln	Val	Tyr
Dog	Val	Gln	His	Pro	Pro	Lys	Ile	Gln	Val	Tyr
Rabbit	Val	Gln	Arg	Ala	Pro	Asn	Val	Gln	Val	Tyr
H-2 associated polypeptide	-	-	Lys	-	Pro	-	-	-	Val	Tyr

	11	12	13	14	15	16	17	18	19	20
Human	Ser	Arg	His	Pro	Ala	Glu	Asn	Gly	Lys	Ser
Dog	Ser	Arg	His	Pro	Ala	Glu	Asn	Gly	Lys	Pro
Rabbit	Ser	Arg	His	Pro	Ala	Glu	Asn	Gly	Lys	Asp
H-2 associated polypeptide	-	-	-	Pro	Pro	-	-	-	Lys	Pro

	21	22	23	24	25	26	27
Human	Asn	Phe	Leu	Asn	Cys	Tyr	Val
Dog	Asn	Phe	Leu	Asn	Cys	Tyr	Val
Rabbit	Asn	Phe	Leu	Asn	Cys	Tyr	Val
H-2 associated polypeptide	-	-	Leu	-	-	Tyr	Val

FIG. 2. Partial amino-acid sequences of β_2-microglobulins from man, dog, and rabbit compared with the H-2 associated polypeptide of mouse. A dash indicates that no one of the six labeled amino acids (alanine, leucine, lysine, proline, tyrosine, and valine) is present at that position. The rat β_2-microglobulin has lysine at position 3 and proline at position 15 (M. D. Poulik, C. Shinnick, and O. Smithies, personal communication). The lysine at position 3 in the mouse β_2-microglobulin has been confirmed by other investigators (B. T. Ballou, D. McKean, and O. Smithies, personal communication). Boxes indicate the corresponding residues are identical to their mouse counterparts. The β_2-microglobulin sequences were obtained from the following sources: human (35), dog (9), and rabbit (36).

tive yields obtained from conventional runs. Furthermore, these high repetitive yields suggest that a single major component is being sequenced. We should be able to detect a 10–20% minor contaminant. Obviously, this statement is qualified by our inability to detect polypeptides with a blocked NH_2-terminus or heterogeneity in residues that were not labeled.

The partial sequence deduced from these data is compared in Fig. 2 to the known sequences of β_2-microglobulins isolated from the urine of other species. The dashes, as well as the identified residues in the mouse H-2-associated polypeptide, represent important data. Since six amino acids were labeled in these preparations (alanine, leucine, lysine, proline, tyrosine, and valine), a dash indicates that the corresponding residue is *not* one of the six labeled residues. Accordingly, residue positions represented by dashes have information and can be used in homology comparisons. For example, at position 1 the mouse H-2-associated polypeptide is *not* valine, hence it is distinct from its rabbit counterpart at this position even though we cannot make a positive identification of the mouse residue at this position.

Amino-Acid Sequence Analysis of the 45,000 Molecular Weight Components. The sequence data from one representative 45,000 molecular weight component is given in Fig. 1B. The repetitive yields for all residues that could be examined ranged between 89 and 94%. Once again this is consistent with the presence of a single major component in each preparation. These sequences are presented in Fig. 3. No evidence for heterogeneity was obtained throughout the 22 positions examined with six labeled amino acids. In summary, the two H-2K and two H-2D gene products that we have examined appear to have *unblocked* NH_2-termini and they appear to represent a single major molecular component.

	1	2	3	4	5	6	7	8	9	10	11
K^b	-	Pro	-	-	Leu	-	Tyr	-	Val	-	Ala
K^k	-	Pro	-	-	Leu	-	Tyr	-	-	-	Ala
D^b	-	Pro	-	-	-	-	Tyr	-	-	-	Ala
D^d	-	-	-	-	Leu	-	Tyr	-	Val	-	Ala

	12	13	14	15	16	17	18	19	20	21	22
K^b	Val	-	-	Pro	-	Leu	-	-	-	-	Tyr
K^k	Val	-	-	Pro	-	Leu	-	Lys	-	-	-
D^b	Val	-	-	Pro	-	Leu	-	-	Pro	-	Tyr
D^d	Val	-	-	Pro	-	-	-	-	Pro	-	Tyr

FIG. 3. Partial amino-acid sequences of the K and D gene products. Dashes at a position indicate a lack of any one of the six labeled amino acids (alanine, leucine, lysine, proline, tyrosine, and valine) and are, accordingly, useful in homology comparisons. For example, the D^b and D^d molecules are identical at position 7 (both have tyrosine) and not identical at position 2 because the D^d molecule does not have proline in this latter position. Boxes indicate identical residues in two or more allelic products.

DISCUSSION

The Small Polypeptide (12,000 Daltons) Is the Mouse β_2-Microglobulin. All 11 residues that we were able to identify are identical to residues at homologous positions in the known sequences of other β_2-microglobulin molecules (Fig. 2). This extensive homology demonstrates unequivocally that the small polypeptide associated with the 45,000 molecular weight components is the mouse β_2-microglobulin. More importantly, the analysis of this partial amino-acid sequence at the level of 100 pmol or less demonstrates the feasibility and reliability of extending these microsequence methods to the analysis of various cell surface molecules available in limited quantities.

The Large Polypeptide (45,000 Daltons) Represents an H-2K or H-2D Gene Product. Control indirect immunoprecipitation experiments using specific alloantisera and radioactively labeled lymphocytes that lack the corresponding H-2K or H-2D specificities have been carried out (7, 21). These experiments suggest that very little radiolabeled protein in the 45,000 molecular weight range is isolated by this procedure unless the specific antigen is present in the immunoprecipitation mixture. It is possible that a small contaminant is being picked up by this procedure and sequenced by our sensitive techniques. This possibility is unlikely because each of the two K and two D gene products is different from all the rest. Therefore, if a contaminant is present, it must vary in accordance with the different allelic gene products. We consider this possibility unlikely. We feel that the serological controls and the presence of amino-acid sequence variability correlated with the differing alleles provide strong support for the supposition that the 45,000 dalton polypeptides are the H-2K or H-2D gene products.

The Four K and D Gene Products Are Homologous to One Another and Probably Have Descended from a Common Ancestral Gene. Of the 8–9 residue positions that are identifiable in each molecule, four are identical in all four proteins (residues 7, 11, 12, and 15). In addition, the K and D molecules share residues at several other positions (e.g., positions 2, 5, 9, 17, and 22). Indeed, a simple statistical cal-

culation can be carried out to determine the probability that any two randomly chosen polypeptides would demonstrate this degree of identity.‡ Using this calculation on pairwise comparisons of the K and D gene products indicates that the probabilities that random polypeptides would exhibit a corresponding degree of identity ranges between 10^{-4} and 8×10^{-8}. Accordingly, the K and D molecules are clearly related. These observations strongly support earlier suppositions that the K and D loci descended from a common ancestral gene (10–13). Furthermore, human transplantation antigens (HL-A antigens) demonstrate 30–40% homology with mouse transplantation antigens over the regions of amino acid sequence that can be compared (24). These data establish that the major histocompatibility complexes of man and mouse (HL-A and H-2, respectively) are homologous genetic regions because of the significant homology demonstrated by their corresponding gene products.

The K and D Gene Products Do Not Appear To Be Homologous to Immunoglobulins Based on Limited Sequence Comparisons. It has been proposed that the immunoglobulin gene families descended from the major transplantation locus. This postulated evolutionary relationship is based on several general features both systems share—their extreme polymorphism, their cell surface location, their role in regulating the immune response, and a variety of general evolutionary arguments (14–16). In addition, the K and D gene products are noncovalently associated with β_2-microglobulin, a molecule homologous to the constant domains of immunoglobulins (see ref. 17). Indeed, preliminary structural studies have suggested the presence of immunoglobulin-like domains in the K and D molecules (25). The limited sequence data presented in Fig. 3 show no significant sequence homology with the various immunoglobulin domains or the β_2-microglobulins. There are a number of possible explanations for this apparent lack of homology. (*1*) The K and D genes are not related to immunoglobulin genes. (*2*) The amino-acid sequence data are insufficient to determine sequence homology or lack thereof. (*3*) The NH_2-terminal portions of the K and D molecules may be particularly variable, perhaps a reflection of some important associated function. Other portions of these molecules may show homologies with immunoglobulins. (*4*) The transplantation antigens may have diverged sufficiently from immunoglobulins to erase any primary amino-acid sequence homologies, but these molecules may retain homology in their three-dimensional structure. Indeed, the V (variable) and C (constant) regions of immunoglobulins demonstrate no primary sequence homologies, but they have very similar three-dimensional structures. In this regard, if the transplantation antigens actually turn out to consist of "heavy" (45,000 dalton) and "light" (12,000 dalton) chains (17) that fold into immunoglobulin-like domains (25), perhaps the contact residues between the two chains would be structurally homologous to their immunoglobulin counterparts from the V_L and V_H domains. Indeed, the six contact residues for the V_L regions (positions 35, 37, 42, 43, 86, and 99) are strikingly conserved in all light chains, as are their seven V_H region counterparts (positions 37, 39, 43, 46, 47, 95, and 108) (26). These postulated homologies can be tested by additional sequence data. Whatever the case may be, the data currently available do not reveal any homology relationship between the transplantation antigens and immunoglobulins.

The Two K Allelic Products Differ from One Another by Multiple Amino-Acid Residues, as Do the Two D Allelic Products. The K products differ by three out of 10 residues and the D products by four of 10 residues (Fig. 3). These constitute 30–40% sequence differences over the limited regions examined. We have recently suggested that alleles (or allotypes) can be divided into two categories (27). Alternative forms of *simple allotypes* segregate in a Mendelian fashion in mating studies and differ by one or a few amino-acid substitutions [e.g., the *Inv* marker of the human κ chain (25)]. In contrast, alternative forms of *complex allotypes* differ by multiple amino-acid residues and *generally* segregate in a Mendelian fashion [e.g., the group a and group b allotypes of the rabbit (see ref. 29)]. By these definitions the K and D alleles, based on limited sequence data, are complex allotypes. The importance of the distinction between simple and complex allotypes lies in the very different types of genetic or evolutionary mechanisms implied (see ref. 27). Simple allotypes are probably coded by alternative alleles at a single structural locus. In contrast, complex allotypes may be explained by one of three genetic models: (*1*) Complex allotypes may evolve by the divergence of alleles at a single genetic locus. If so, intense selective pressures are required to fix many substitutions in relatively short periods of evolutionary time. (*2*) Complex allotypes may evolve by gene duplication, mutational divergence, and subsequent crossing-over events that contract the gene number to one. In different populations (e.g., inbred strains) different genes could remain. (*3*) Complex allotypes may evolve by gene duplication with a control mechanism that permits them to be expressed so they mimic a Mendelian pattern of genetic segregation. The important point is that the K and D alleles may have evolved by any one of these three mechanisms and various experimental approaches will allow one to distinguish among the possibilities. Whatever the genetic explanation for these complex allotypes, the extensive polymorphism of the major transplantation locus of the mouse does reflect extensive sequence differences among the corresponding "alleles" at the K and D regions.

The K Products Cannot Be Distinguished from the D Products on the Basis of Limited Sequence Data. The D gene products do not appear to be significantly more closely related to one another than to the K gene products. For example, the D^d gene product shows 60% homology with K^b, 55% homology with K^k, and 55% homology with its allelic counterpart, the D^b gene product (Fig. 3). Likewise, apart from the proline residues at position 20, no amino acids are restricted to only the K or the D gene products (Fig. 3). This lack of "D-ness" or "K-ness" is perhaps the most surprising observation in these data. The existence of two subloci (LA and 4) in the major histocompatibility complex of man (see ref. 30) that are homologous to the K and D regions of the mouse suggests that the gene duplication event(s) which created these regions occurred prior to the divergence of these species 75 million years ago. If the allelic forms of the K or D region arose subsequent to the divergence of the mammalian evolutionary lines, the alleles of one region

‡ This simple calculation assumes that any of the twenty different amino acids are equally likely to appear at any of the residue positions. Hence the probability that any two random sequences are as closely related as indicated in Fig. 3 is given by

$$P_n(m) = \binom{n}{m} \left(\frac{19}{20}\right)^{n-m}$$

where n is the number of positions at which amino acids can be compared and m is the number of residue identities.

should be more closely related to one another than to those of the second region. We can offer three types of explanations for our apparent inability to distinguish the K and D allelic products. (1) Perhaps our limited partial sequence data are misleading in this regard. (2) The NH_2-terminal regions of these molecules may not have any important function or they may have a function compatible with great variability and thus are capable of accepting many mutations. Other regions of these molecules may show "K-ness" and "D-ness." (3) If neither of these trivial explanations is correct, unusual genetic mechanisms will have to be considered (e.g., the transplantation antigens may share a pool of "V genes" or nonhomologous recombination or gene conversion may permit variants to be exchanged between the K and D loci). Additional amino-acid sequence data should allow us to begin to distinguish among these alternatives.

Chromosome 17 of the Mouse Codes for a Variety of Cell Surface Molecules That May Be Homologous to One Another. Chromosome 17 of the mouse codes for at least five different kinds of cell surface molecules: the T/t antigens, the K antigens, the I antigens, the D antigens, and the TL antigens (see ref. 2). Recently it has been suggested that the T (31) and TL (32) antigens are homologous to the K and D gene products on the basis of similar molecular weights and their association with β_2-microglobulin. If confirmed by structural studies, this suggests that the corresponding genes may have diverged from a common ancestor. Furthermore, clusters or families of genes appear to be present which code for protein with related structures and functions. These multigene families include the T/t, I, and S regions that are respectively associated with embryological development (33), regulation of the immune response (3), and complement components (see refs. 4 and 5). It will be interesting to determine whether these multigene families share strategies for information storage, information expression and information evolution similar to those seen in the antibody gene families (see ref. 34).

The approach we have taken to the analysis of the K and D gene products should lend itself to the study of other membrane proteins. In time these studies should provide insights into the fascinating organizational, regulatory, evolutionary, and functional mysteries of many complex eukaryotic systems.

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