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Immunology

Structure of the heavy chain of the H-2K^k histocompatibility antigen

(CNBr fragments/radiochemical sequence analyses/tunicamycin/phosphorylation)

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ABSTRACT **We have used radiochemical techniques to characterize the heavy chain (M_r 46,000) of the murine H-2K^k histocompatibility antigen in terms of six fragments (I–VI) obtained after cleavage of the polypeptide chain with CNBr. The tentative order of the fragments, which account for more than 90% of the heavy chain, was assigned by radiochemical sequence analysis of the intact heavy chain and of each purified CNBr fragment and by analysis of the CNBr fragments obtained from the large papain fragment of the heavy chain. Treatment of cells with tunicamycin yielded H-2 molecules with heavy chains of molecular weight 40,000, suggesting that the carbohydrate moieties have a combined molecular weight of approximately 6000. CNBr cleavage of H-2K^k heavy chains labeled with [^{3}H]fucose indicated that the carbohydrate moieties are located on fragments II and IV. Incubation of cells with $^{32}PO_4$ gave H-2 molecules with radioactive phosphoserine in the carboxyl-terminal CNBr fragment (VI) of the heavy chain and in the fraction containing β_2-microglobulin. Sequence analysis of each CNBr fragment intrinsically labeled with ^{3}H- and ^{35}S-labeled amino acids identified a total of 87 residues in the H-2K^k heavy chain. The sequence closely resembles that of the H-2K^b molecule, and the 11 differences are scattered throughout the polypeptide chain. Comparison with HLA sequences indicates that the two allelic H-2 sequences are more closely related to each other (88% identity) than either is to the HLA-B7 or A2 antigens (≈70%). Similarly, the nonallelic HLA antigens are more closely related to each other (83%) than either is to the H-2K^k or H-2K^b molecules.**

The major histocompatibility antigens of man (HLA) and mouse (H-2) are a genetically polymorphic class of cell surface proteins that appear to be involved in a variety of immunological phenomena (for review see refs. 1 and 2). The heavy chains (M_r 46,000) of these molecules are integral membrane glycoproteins that bear the antigenic specificities. The noncovalently associated light chain, β_2-microglobulin (M_r 12,000), is a relatively invariant protein characterized by its homology with the constant region domains of immunoglobulins (3). HLA and H-2 antigens can be released intact from the cell surface by treatment with detergents or by treatment with papain (4), which cleaves the heavy chain near the carboxyl terminus (5, 6). Recent data (7) have indicated that the HLA heavy chain, but not its papain fragment, is phosphorylated.

The partial amino acid sequences of the amino-terminal portions of a variety of H-2 (5, 6, 8–10) and HLA heavy chains (11, 12) have been determined. Extensive structural data also have been obtained by conventional methods for most of the HLA-B7 heavy chain and for large segments of the nonallelic HLA-A2 molecule (13–16). Recently, the partial structure of the papain fragment of the H-2K^b heavy chain has been determined by using radiochemical methods (17, 18).

We report here the characterization of six fragments that we have isolated after cleavage of the intact heavy chain of the H-2K^k molecule with CNBr. Partial radiochemical sequence analysis and localization of the sugar moieties and phosphate groups indicate that the H-2K^k molecule closely resembles the H-2K^b protein and is similar to the HLA-B7 and A2 molecules.

MATERIALS AND METHODS

R1 lymphoma cells were labeled with ^{3}H-, ^{14}C-, and ^{35}S-labeled amino acids, [^{3}H]fucose, and ^{125}I in Dulbecco's modified Eagle's medium (10^7 cells per ml) as described (5). Cells were labeled with [^{32}P]orthophosphate by preincubation for 1 hr in phosphate-free medium followed by resuspension in fresh medium containing 100 μCi (3.7×10^6 becquerels) of [^{32}P]orthophosphate per ml and incubation for 4 hr. Tunicamycin (1 μg/ml) treated R1 and EL4 cells were grown at lower density (2×10^6 cells per ml) for 24 hr in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Nonidet P-40 (NP40) extracts (5) were partially purified by affinity chromatography on columns of *Lens culinaris* (lentil) lectin coupled to Sepharose (19), and the H-2 antigens were purified by immunoprecipitation. Papain fragments were prepared in an identical fashion except that the NP40 extract was incubated with the enzyme for 15 min at 23°C.

After immunoprecipitation, washed precipitates were redissolved in 7.5 M guanidine·HCl/1.0 M Tris·HCl/1 mM Na_2EDTA/0.1 M dithiothreitol, pH 8.0, and incubated for 3 hr at 37°C. The cysteines were then alkylated with iodoacetamide (0.25 M) for an additional 15 min. Ovalbumin and cytochrome *c* (3 mg each) were added as carrier proteins and the reaction mixture was loaded onto a column of Sepharose CL6B. Fractions from the column were desalted on Sephadex G-25 in 10% (wt/wt) formic acid and lyophilized.

Proteins were treated with CNBr (125 mg/ml) in 70% (wt/wt) formic acid for 10 hr at room temperature. After drying under reduced pressure, the reaction products were separated on Sephacryl S-200 in 6 M guanidine. Pooled fractions were desalted as described above.

To detect ^{32}P-labeled residues, dried samples were redissolved in 6 M HCl and hydrolyzed under reduced pressure at 105°C for 1 hr. Hydrolyzates were analyzed by paper electrophoresis with authentic phosphoserine, phosphothreonine, and phosphotyrosine (20) at pH 1.9 (21).

Sequence analyses were carried out in a Beckman 890C protein sequencer, using 0.28 M Quadrol buffer and a single cleavage program (repetitive yields 94–97%). For samples containing multiple ^{3}H-labeled amino acids, individual phenylthiohydantoin derivatives of amino acids were separated by high-pressure liquid chromatography.

For analysis of the intact chain, the sequencer was stopped after 18 cycles and the cup was allowed to cool to room temperature. Aminopeptidase M (Rohm and Haas) (50 μl of a solution of 3 mg/ml) in 0.1 M triethylamine acetate, pH 7.5, was

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Abbreviations: NP40, Nonidet P-40; gp70, viral glycoprotein of M_r 70,000.

added and the cup was spun at room temperature. After 4 hr an additional 50 μl of the enzyme solution was added and the reaction was allowed to continue for 12 hr. The sample was dried by evaporation and the cup was returned to 55°C. The sequencer was then restarted.

Polyacrylamide gel electrophoresis was carried out in NaDodSO$_4$ (22) or NaDodSO$_4$/urea (23) on slabs or rods.

RESULTS

Isolation of H-2K^k Heavy Chain. H-2K^k antigens were initially purified from extracts of radiolabeled R1 lymphoma cells by affinity chromatography on columns of lentil lectin to remove actin and much of the viral glycoprotein gp70 (24). After immunoprecipitation, gel filtration of H-2K^k molecules on Sepharose CL6B in 6 M guanidine (Fig. 1) removed any remaining gp70, which appeared as a peak or shoulder preceding the H-2 heavy chain, and separated the H-2K^k heavy chain from β_2-microglobulin.

CNBr Fragments. Fig. 2*A* is a representative profile obtained when the H-2K^k heavy chain containing ^{3}H-labeled amino acids was treated with CNBr and subjected to gel filtration on Sephacryl S-200. The six unique CNBr fragments (denoted I–VI) described in this report correspond to material in fractions e through j; fractions a through d were judged by their molecular weight and by partial sequence analysis to contain the uncleaved polypeptide chain or products of incomplete CNBr degradation. Labeling with different tritiated amino acids gave similar profiles in that the peaks were at the same positions, but differed in height, presumably reflecting the amino acid composition of each fragment. The CNBr fragments differed sufficiently from each other in size so that gel filtration was the only method necessary to achieve sufficient purity for sequence analysis of all six fragments. Each fragment, however, was further purified by gel filtration on the same column (Fig. 2 *B–E*); in order to minimize the number of columns required, fractions that contained fragments of substantially different molecular weight (for example, peaks a and e in Fig. 2*B*) were pooled for the second gel filtration.

The molecular weight of each fragment (Table 1) was estimated by comparing the position of its elution from the column to the positions of proteins of known molecular weight. The size

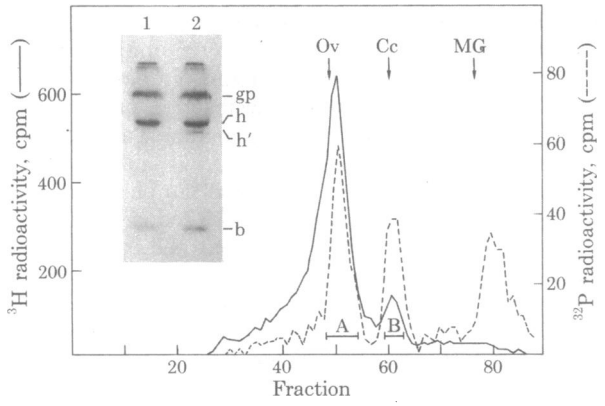

FIG. 1. Isolation of H-2K^k heavy chains. Redissolved immunoprecipitates were loaded on a column (2.25 × 130 cm) of Sepharose CL6B in 6 M guanidine·HCl/0.1 M Tris·HCl, pH 8.6. Fractions contained 8 ml; 25-μl aliquots were used for determination of radioactivity. Horizontal bars indicate fractions pooled for: A, H-2K^k heavy chains; B, β_2-microglobulin. Arrows indicate the positions of markers: Ov, ovalbumin (M_r 43,000); Cc, cytochrome *c* (M_r 11,700); MG, methyl green. (*Inset*) NaDodSO$_4$/polyacrylamide gel electrophoresis of immunoprecipitation of H-2K^k antigen from R1 cells (lane 1) and R1 cells treated with tunicamycin (lane 2); gp, gp70; h, H-2K^k; h′, unglycosylated H-2K^k; b, β_2-microglobulin.

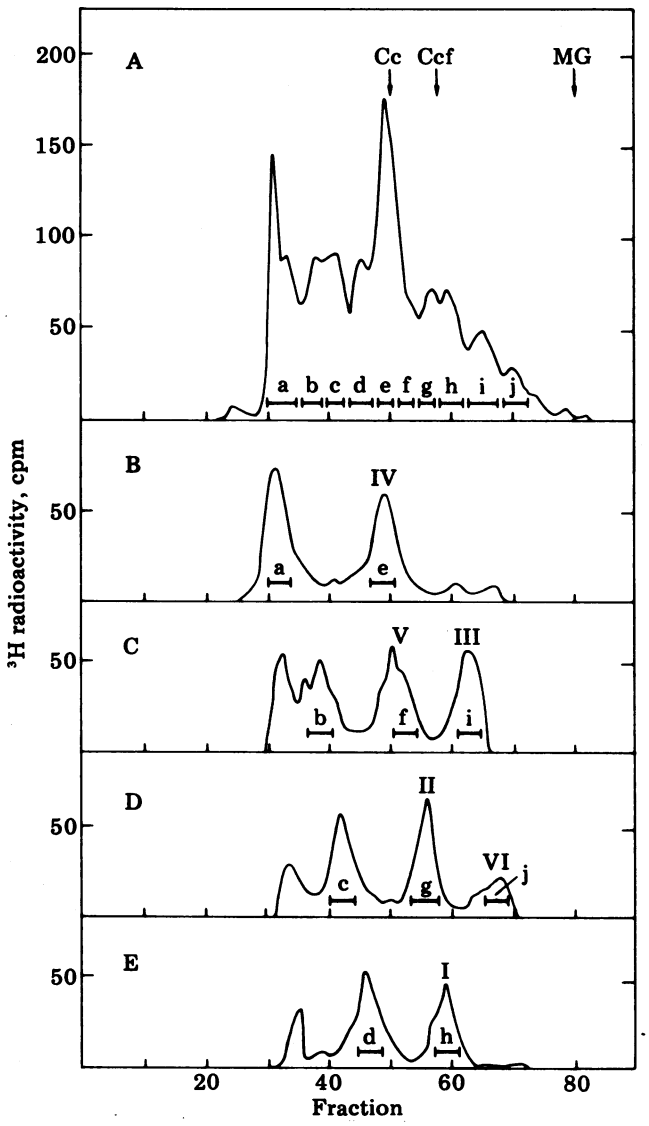

FIG. 2. Separation of CNBr fragments of the H-2K^k heavy chain. After CNBr treatment, fragments (shown here for [^{3}H]threonine) were chromatographed on a column (1.75 × 140 cm) of Sephacryl S-200 in 6 M guanidine·HCl/0.1 M Tris·HCl, pH 8.6. Fractions contained 3.5 ml; 60 μl was used for determination of radioactivity. Individually pooled fractions a–j from the initial separation (*A*) were run on the same column (B–E); I–VI indicate CNBr fragments identified in each fraction. Arrows denote the positions of markers: Cc, cytochrome *c* (M_r 11,700); Ccf, the heme-containing CNBr fragment of cytochrome *c* (M_r 7760); MG, methyl green.

and purity of the fragments also were assessed by NaDodSO$_4$/urea gel electrophoresis (23).

Carbohydrate Moieties. To determine the amount of carbohydrate on the H-2K^k heavy chain, R1 cells were treated with

Table 1. Characteristics of the major CNBr fragments of H-2K^k

Fragment	M_r*	Carbohydrate ([^{3}H]fucose)	Phosphate (^{32}PO$_4$)
I	5,500	–	–
II	7,100	+	–
III	3,900	–	–
IV	12,400	+	–
V	8,600	–	–
VI	2,800	–	+

* Based on mobility on NaDodSO$_4$/urea gels.

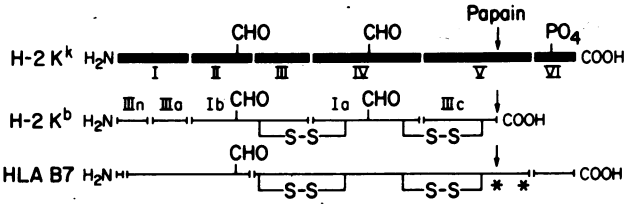

FIG. 3. Tentative model of the H-2K^k heavy chain compared to the H-2K^b (17) and HLA (13) heavy chains. Horizontal bars indicate the size of the CNBr fragments produced by cleavage at methionyl residues. Asterisks mark the hydrophobic portion of the HLA-B7 antigen that is thought to cross the lipid bilayer (15). CHO denotes carbohydrate; PO_4, the phosphate moiety of phosphoserine residues.

tunicamycin to inhibit glycosylation of the polypeptide chain (25). After treatment of the cells with the drug for 24 hr, immunoprecipitation resulted in the isolation of a large amount of the normal heavy chain (M_r 46,000) and a lower molecular weight (M_r 40,000) crossreacting polypeptide (Fig. 1, *Inset*) that we assume is an unglycosylated form of the heavy chain (h'). Anti-gp70 does not precipitate this species. Similar results were obtained when EL4 cells were treated with tunicamycin and the H-2K^b molecules were precipitated with specific alloantisera. R1 cells and EL4 cells, however, appeared to differ in their sensitivity to tunicamycin in that immunoprecipitation of the H-2K^b molecule gave more of the unglycosylated form of the heavy chain. These results suggest that the molecular weight of the carbohydrate on the H-2K^k molecule, like that on H-2K^b (26), is 6000.

To locate the carbohydrate moieties within the H-2K^k protein, R1 cells were simultaneously labeled with [^{3}H]fucose and a mixture of ^{14}C-labeled amino acids. The H-2K^k heavy chain was then isolated and cleaved with CNBr, and the fragments were separated on Sephacryl S-200 (data not shown). The distribution of the ^{14}C label was similar to that obtained when the protein was labeled with ^{3}H-labeled amino acids (see Fig. 2*A*). The [^{3}H]fucose coeluted with the material in fractions e and g, indicating that fragments II and IV contain carbohydrate. [^{3}H]Fucose also was present in fractions a through d, consistent with our assumption that these fractions contain uncleaved or partially cleaved molecules and the fact that fragments II and IV would be contained in any such material. Fragments II and IV contained approximately equivalent amounts of [^{3}H]fucose, so we tentatively assume that there are two carbohydrate moieties of similar size on the H-2K^k heavy chain, as is the case for the H-2K^b molecule (17).

Phosphorylation. Recent experiments have indicated that both HLA-B7 and -A2 heavy chains can be phosphorylated *in vivo* (7). We were able to demonstrate *in vivo* labeling of the H-2K^k molecule with $^{32}PO_4$, using R1 cells only after modifying the medium, purifying the NP40 extract on a lentil lectin column, and washing the immunoprecipitates thoroughly before gel filtration on Sepharose CL6B (Fig. 1). ^{32}P eluted with the heavy chain (fraction A) and β_2-microglobulin (fraction B). Partial hydrolysis of each fraction, followed by paper electrophoresis (21), demonstrated that both fractions contained ^{32}P-labeled phosphoserine but not significant amounts of either phosphothreonine or phosphotyrosine. The large fragment of the papain-cleaved heavy chain does not contain ^{32}P under the same conditions. This result indicates that the phosphoserine in the heavy chain, like that in the HLA antigens (7), is on the carboxyl-terminal side of the papain cleavage site.

To locate more precisely the phosphate moiety in the H-2K^k heavy chain, cells were labeled with $^{32}PO_4$ and [^{3}H]tyrosine, and the isolated heavy chain was cleaved with CNBr. On gel filtration on Sephacryl S-200, the ^{32}P label eluted as a single symmetrical peak, superimposed on fragment VI.

Order of CNBr Fragments. The tentative order of the CNBr fragments (I through VI, Fig. 3) was established by radiochemical sequence analyses of the intact molecule and each CNBr fragment, and by treating the papain fragment of the heavy chain with CNBr.

The intact heavy chain and each CNBr fragment intrinsically labeled with ^{3}H- and ^{35}S-labeled amino acids were subjected to automatic sequence analysis. The results obtained for the six CNBr fragments are summarized in Fig. 4. Assignments of an amino acid were made at each position where a peak of radioactivity was consistent with the repetitive yield curve for that determination. Occasionally small peaks were seen that were off the repetitive yield curve; in most cases, such peaks were identified as authentic residues in other fragments. The H-2K^k molecule contains no methionine in the first 30 residues (27). To locate the first methioninyl residue in the heavy chain, the sequence of the intact molecule labeled with [^{35}S]methionine and [^{3}H]tyrosine was determined, using aminopeptidase M after 18 cycles to realign amino-terminal ends that might be frayed by incomplete chemical cleavage. In agreement with earlier results (5, 27), tyrosines were found at positions 7 and 27, and an additional tyrosine was detected at position 45. The first peak of ^{35}S was detected at position 52, indicating that the first methionine is at this position. Consistent with this result, fragment I (M_r 6000) had the same sequence as the amino-terminal portion of the H-2K^k molecule (5, 27), placing it at the amino terminus of the peptide chain.

The remaining fragments tentatively could be positioned by comparing their partial sequences (Fig. 4) with those of the H-2K^b (18) and HLA-B7 (14) and -A2 (15) heavy chains (Fig. 5). In addition to their having sequence homologies, fragments II and IV are glycosylated, as are the corresponding fragments in H-2K^b (17). In most cases, the comparisons allow unambiguous placement of the fragments of H-2K^k.

Additional support for placing fragments V and VI was obtained by analysis of the CNBr fragments from the papain fragment of the H-2 molecule. Gel filtration of the products of CNBr cleavage (data not shown) gave a profile similar to that for the CNBr fragments of the whole heavy chain (Fig. 2*A*) except that peaks f (fragment V) and j (fragment VI) had decreased and peak h had increased significantly. These results suggest that: fragment V contains the papain cleavage site(s); the carboxyl-terminal portion of fragment V and all of fragment VI are missing; and the remainder of fragment V coelutes with

	1	5	10	15	20	25	30	35	42	45
I	— — HIS — LEU ARG TYR PHE HIS THR — VAL — ARG — — LEU — LYS — ARG PHE ILE — VAL — TYR — — — THR — PHE — ARG PHE ... ... TYR ...									
II	— — VAL — — — TYR — — ARG — THR — — ALA LYS — — — — — — PHE ARG VAL — — ARG THR —(LEU)ARG TYR TYR — — — ... THR ...									
III	TYR(ILE)— — — — — — — —(LYS)LEU LEU — — TYR — — TYR — — — — — — — TYR(ILE)— — — — — —(LEU)...									
IV	ALA ALA LEU ILE THR LYS HIS LYS TRP — VAL — — — — — — ARG — ARG — TYR LEU — — THR — VAL — — LEU ARG ARG ...									
V	— — VAL — THR ARG — — — — — — THR PHE — LYS TRP — — — — — — — — — — — — —(TYR)...									
VI	ARG ARG ARG — — — — LYS — — — TYR(ALA)LEU ALA ...									

FIG. 4. Summary of radiochemical sequence data for the CNBr fragments of the H-2K^k heavy chain. A total of 82 residues were identified in fragments I–VI. Assignment of the seven residues in parentheses are considered tentative; — indicates a position where no amino acid could be assigned.

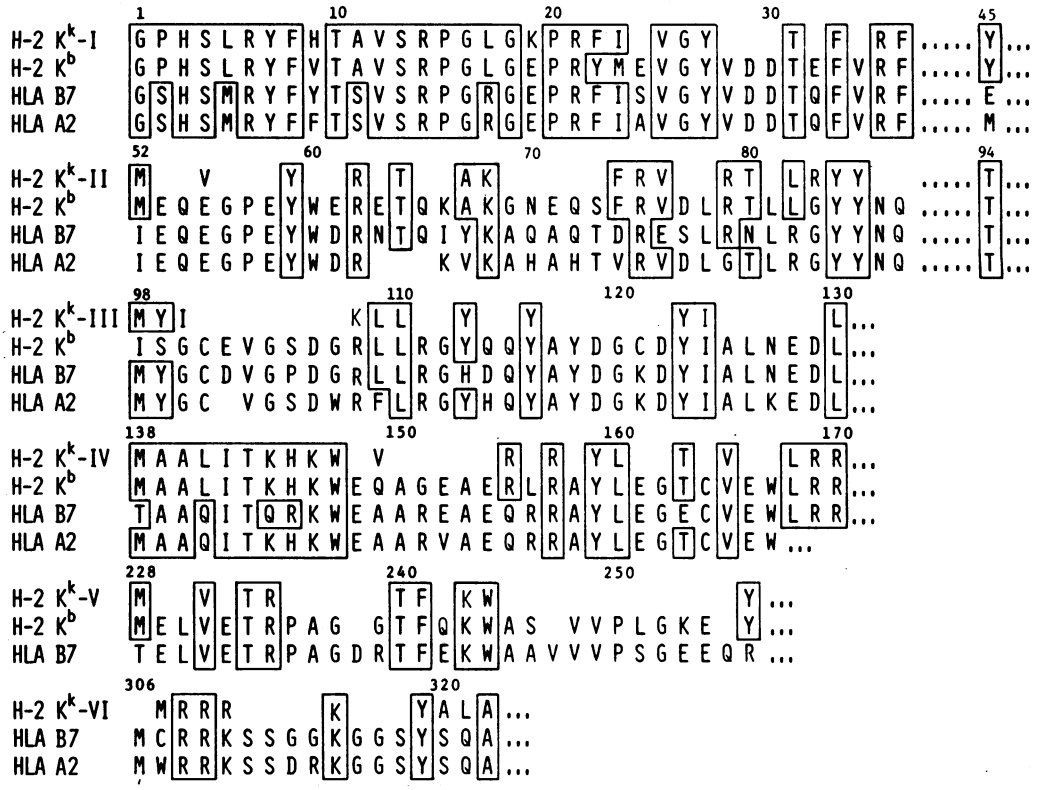

FIG. 5. Comparison of the amino acid sequences of H-2 and HLA heavy chains. The standard one-letter code is used (28). The amino-terminal sequence data for the CNBr fragments (I–VI) of the H-2K^k antigen are compared to corresponding portions of the H-2K^b (18), HLA-B7 (14), and HLA-A2 (15) antigens. Methionyl residues were placed before the sequences of H-2K^k fragments II–VI because of the specificity of CNBr. Numbering is from HLA-B7. Glycyl, seryl, and prolyl residues in fragment I are from previous studies (10, 27). Identical residues are enclosed in boxes.

fragment I. Consistent with this interpretation, radiochemical sequence analysis of the mixture in the larger fraction h gave the combined sequences of fragments I and V (see below and Fig. 3). In addition the phosphate moiety, which is found exclusively in fragment VI, was absent from the papain fragment.

DISCUSSION

An outstanding feature of the major transplantation antigens is their extensive genetic polymorphism (1). Nevertheless, the molecules are extremely similar to each other in overall structure and in amino acid sequence. The resemblance is readily seen on comparing our model of the H-2K^k protein (Fig. 3) with the H-2K^b papain fragment (17) and the HLA-B7 (13) heavy chain.

Comparisons* of the amino acid sequences (Fig. 5) again emphasize the similarities. Of the 88 residues in the H-2K^k protein that have a known counterpart in H-2K^b, 77 are identical. A more distant relationship is seen between the H-2K^k molecule and the HLA-B7 (66 identical out of 97 comparable residues) and the HLA-A2 antigens (61 out of 84 residues). The H-2K^b heavy chain resembles HLA-B7 and -A2 molecules to about the same extent as H-2K^k, and, at the same positions, HLA-B7 and HLA-A2 antigens are identical to each other in 70 out of 84 residues. Overall, the comparisons indicate that of the 97 positions that can be compared, molecules from the same species are identical in about 85% of the residues, whereas antigens from different species are identical in only about 70% of the residues, despite the fact that the H-2K^k and K^b heavy chains are allelic products of the same locus and the HLA-B7 and -A2 polypeptides are the products of separate loci. This result is similar to earlier comparisons of the amino-terminal sequences of H-2 and HLA antigens (1, 2), which raised the possibility that the histocompatibility loci may have diverged in evolution after the lines leading to humans and mice, or that the classical H-2 and HLA genes are regulatory rather than structural genes (29).

Comparison of the sequences of H-2K^k and H-2K^b heavy chains reveals that there are a minimum of 11 amino acid substitutions and, consistent with peptide mapping studies (30), these occur throughout the sequence at least up to position 149. Beyond this position, all residues identified so far are identical, although few residues are available for comparison in this region.

Previous comparison (31) of the H-2K^b heavy chain with HLA antigens indicated a clustering of differences between residues 60 and 80, where the HLA-B7 and -A2 molecules also differ considerably. Surprisingly, the H-2K^k and K^b antigens are identical in all 9 of the positions so far determined in this area, although we detect no lysine at position 66 of H-2K^k and we have identified two positions (55 and 83) immediately outside of this region where H-2K^k differs from H-2K^b and the HLA antigens. The H-2K^k and K^b antigens may be less variable in this region because they are products of allelic genes, but we cannot exclude the possibility that some of these changes are species-specific differences between HLA and H-2 antigens.

The H-2 antigens have two carbohydrate groups (ref. 26, Fig. 3), whereas the HLA antigens have only one; the position of the carbohydrate moiety in the HLA antigens, however, is comparable to one of those found in the H-2 antigens. The function of the carbohydrate is unknown and there is, as yet, no way to ascertain the possible significance of the fact that the H-2 molecules have an additional saccharide moiety.

* In all of our comparisons, we exclude negative differences—i.e., positions where we assign no residue but an amino acid tested by us was assigned in the H-2K^b or HLA sequences.

Our studies with tunicamycin support the earlier conclusion that the carbohydrate groups do not serve as major alloantigenic determinants (26), because both H-2K^k and H-2K^b alloantisera appear to recognize molecules that lack the bulk of the carbohydrate. The fact that the alloantisera can recognize unglycosylated H-2 molecules also suggests that the carbohydrate may not play a major role in determining the three-dimensional folding of the molecule. In support of this hypothesis, studies of HLA antigens have suggested that glycosylation is not required for interaction of the heavy chain with β_2-microglobulin or for its expression at the cell surface (32).

In comparison to HLA antigens, less is known about the portions of the H-2 molecule that are associated with the cell membrane and exposed on the cytoplasmic side of the membrane. The major papain cleavage site, however, does appear to be similar (Fig. 3), and the region of the H-2K^k molecule represented by our fragment VI has considerable sequence homology with the portion of HLA molecule that is thought to be localized on the cytoplasmic side of the membrane (15).

The observation that the cytoplasmic portions of the transmembrane proteins can be phosphorylated raises speculation about the role of phosphorylation in the interaction of such proteins with cytoskeletal components and other cytoplasmic elements and the role of such interactions in the regulation of cell function and growth (33). Any estimate of the significance or extent of phosphorylation of the H-2 heavy chain, however, must be viewed with caution. The possibility that the phosphorylation of the H-2 and HLA molecules, like the formation of disulfide bonds between heavy chains (5, 11), occurs after the lysis of the cells with detergent has not been eliminated. Moreover, our observation that material in the β_2-microglobulin fraction also contains ^{32}P suggests that phosphorylation may occur extracellularly, because β_2-microglobulin is not a transmembrane protein. We have not yet shown that the phosphoserine in the β_2-microglobulin fraction is derived from β_2-microglobulin on the cell surface rather than from intracellular β_2-microglobulin or a fragment of the H-2 heavy chain. Electrophoresis of various β_2-microglobulins (34, 35), however, has indicated that there are distinct forms of the molecule; such forms could arise by phosphorylation or dephosphorylation.

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