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Biochemistry

Subunit structure, cell surface orientation, and partial amino-acid sequences of murine histocompatibility antigens

(detergent and papain solubilized H-2 antigens/molecular weight/H-2 antigen model/H-2 haplotypes b,d,k)

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ABSTRACT **Detergent and papain solubilized murine histocompatibility (H-2) antigens have been compared by gel exclusion chromatography, ultracentrifugation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and amino-acid sequence analysis. From these data, we propose a molecular model for the H-2 antigens that includes the size and arrangement of the subunits on the cell surface and in solution, and we provide evidence for the orientation of these molecules on the cell surface. Detergent solubilized H-2 antigens (molecular weight 116,000) consist of two disulfide-linked heavy chains (46,000 daltons) and two noncovalently associated light chains (12,000 daltons). Alkylation with iodoacetamide prior to extraction prevented the formation of a disulfide linkage between the two heavy chains. A water-soluble 51,000-dalton molecule (F_s) consisting of a 39,000-dalton fragment (F_H) of the heavy chain and one intact light chain was obtained by papain digestion of cells or detergent extracts. Therefore, the disulfide linkage between the heavy chains is located in the remaining membrane-associated portion (F_m). Amino-acid sequence analysis of the F_H fragment of H-2K^b by radiochemical techniques showed that it is identical to the detergent solubilized H-2K^b heavy chain in eight positions for the three amino acids tested. These data indicate that the fragment F_H derives from the amino-terminus of the heavy chain and suggest that it projects outward from the cell surface, while the carboxyl-terminal region is associated with the plasma membrane. The described amino-terminal sequence data have been found constant in H-2K^b, H-2K^d, H-2K^k, H-2D^b, and H-2D^d gene products. These data support the hypothesis that the K and D products of the major histocompatibility antigen complex have evolved by gene duplication.**

The importance of histocompatibility antigens in the rejection of allografts has been established, but the molecular structure and biological function of these cell-surface glycoproteins are largely unknown. Recent studies support the notion that these molecules function in the discrimination of altered self antigens, e.g., in distinguishing normal cells from virally infected or transformed cells (1–3). A clue to the role of histocompatibility antigens in this process is provided by observations suggesting that the proteins interact with viral or tumor antigens to form structures that can be recognized by syngeneic cytotoxic T lymphocytes. Our understanding of the mechanisms of these interactions depends upon knowledge of the detailed chemical structure of the histocompatibility antigens.

Human (HL-A) and murine (H-2) histocompatibility antigens consist of two polypeptide chains, one heavy chain [molecular weight (M_r) $\approx$ 50,000] bearing the antigenic specificity and the carbohydrate (4) and a small polypeptide (M_r 12,000) that has been identified as β_2-microglobulin (5–7). The amino-acid sequence of β_2-microglobulin has been shown to be homologous to the constant regions of immunoglobulin light and heavy chains, raising the possibility of an evolutionary relationship between the immunoglobulins and the histocompatibility antigens (8, 9). In support of this hypothesis recent reports indicate that both H-2 and HL-A antigens resemble immunoglobulins in that they appear to be molecules composed of two heavy chains and two light chains (10, 11).

We have carried out an analysis of the molecular organization of H-2 antigens on the cell surface and their physicochemical properties in solution. Partial amino-terminal sequences of heavy chains of different haplotypes were compared in order to obtain some insight into the nature and organization of the antigenic polymorphism. On the basis of these and other studies, we propose a model for the H-2 antigens which includes the arrangement of the polypeptide chains, the location of interactions between the chains, and the orientation of these proteins on the cell surface. The structural homologies found in the amino-terminal sequences of five different H-2 heavy chains allow conclusions about the origin and the diversity of histocompatibility antigens.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; M_r, molecular weight; NP 40, Nonidet P-40; DOC, sodium desoxycholate.

MATERIALS AND METHODS

Solubilization of H-2 Antigens. Splenic lymphocytes (12) from inbred mice strains (BALB/c, DBA/2J, AKR/J, C57BL/10J, and C57BL/6J obtained from Jackson Laboratories) and lymphoma cells (P388, EL4, and MOPC 21, obtained from the Salk Institute) were labeled by the lactoperoxidase procedure (13) using 1 mCi of ^{131}I or ^{125}I per 2×10^8 cells. Alternatively, cells were labeled by incubation (8×10^6 cells per ml) in minimal essential medium (GIBCO) containing 100 μCi/ml of ^3H-, ^{14}C-, or ^{35}S-labeled amino acids (New England Nuclear) for 4–6 hr at 37°. After washing, the cells were extracted with 0.5% Nonidet P-40 (NP 40). H-2 antigens were isolated from the extracts by indirect immune precipitation (14). Spleen cell membrane fractions were obtained as a pellet by centrifugation (105,000 $\times$ *g*, 60 min) of the supernatant from labeled cells that had been repeatedly frozen and thawed in phosphate-buffered saline and centrifuged at 400 $\times$ *g* for 5 min. Isolated membranes were extracted with 0.5% NP 40 or 0.5% sodium desoxycholate (DOC) in 0.01 M Tris·HCl buffer, pH 8.0, 0.15 M NaCl, and centrifuged for 60 min at 105,000 $\times$ *g*. The clear supernatant was used for gel chromatography and sucrose density gradient centrifugation. Alkylation of cells or membrane fractions was performed using 0.01 M iodoacetamide in 0.01 M Tris·HCl buffer, pH 8.0, 0.15 M NaCl. Water-soluble antigens were prepared by papain treatment of cells or deter-

gent extracts using 2.0 mg of papain (Sigma) for 2×10^8 cells in 1 ml of phosphate-buffered saline or for 1 ml of detergent extract in the presence of 2 mM cysteine for 1 hr at 37°. Proteolysis was terminated by addition of iodoacetamide to a final concentration of 5 mM.

Assay of H-2 Antigens. H-2 alloantisera were prepared by multiple intraperitoneal injections of cell suspensions of spleen, thymus, and lymph node in the following strain combinations: anti-H-2D^b, (A/J × B10.D2/oSn)F1 anti-B10.A(2R)/SgSn; anti-H-2K^b, (A/J × B10.D2/oSn)F1 anti-B10.A(5R)SgSn; anti-H-2D^d, (AKR.M × C57BL/10J)F1 anti-B10.A/SgSn; anti-H-2K^d, (C3H/J × C57BL/10J)F1 anti-C3H.OH/Sn; anti-H-2K^k(B10.D2/oSn × C3H.NB)F1 anti-B10.R III(7INS). All mice were obtained from Jackson Laboratories. Antigenic activity was assayed by inhibition of complement-dependent cytotoxicity using ^{51}Cr-labeled splenic lymphocytes (15).

Molecular Weight Determinations. Immune precipitates were dissolved in 2% sodium dodecyl sulfate ($NaDodSO_4$) and subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis (16, 17). Gels were sliced and the fractions analyzed for radioactivity. The following proteins, labeled with ^{125}I or ^{131}I using chloramine T (18), were used as standards: MOPC 104 E IgM (Bionetics), M_r of μ chain = 73,000; MOPC 21 IgG (Bionetics), M_r of γ chain = 50,000 (19), M_r of light chain = 23,000 (20); bovine serum albumin (Sigma), M_r 68,000; ovalbumin (Sigma), M_r 43,000; carboxypeptidase B (Worthington), M_r 34,000; chymotrypsinogen (Worthington), M_r 23,500; and cytochrome *c* (Sigma), M_r 12,400. Gel chromatography and sucrose density gradient centrifugation were performed as described in the figure legends. Dextran blue (Pharmacia) and phenol red were used to indicate the void and the total bed volume of Sephadex columns. Diffusion coefficients (21, 22) and sedimentation coefficients (23) were used to calculate molecular weights (22).

Amino-Acid Sequence Determination. Heavy chains from reduced and alkylated H-2 antigens individually labeled with ^{3}H- or ^{35}S-labeled amino acids were isolated from immune precipitates by preparative $NaDodSO_4$-polyacrylamide gel electrophoresis using H-2 antigens labeled with 5-dimethylamino-1-naphthalene sulfonyl chloride as fluorescent markers (manuscript in preparation). Antigens were electrophoretically eluted from gel slices, dialyzed against water, and lyophilized. Excess $NaDodSO_4$ was removed by extraction with methanol. Radiosequence analysis (24) was performed by a manual procedure (25) modified to obtain phenyl thiohydantoin amino acids and automatically by a Beckman model 890C sequencer and the Quadrol double cleavage program (Beckman Instruments). An internal standard, [^{35}S]methionine-labeled MOPC 21 light chain (see ref. 20 for sequence data) isolated by immune precipitation and preparative $NaDodSO_4$-polyacrylamide gel electrophoresis, and 0.4–2.0 mg of succinylated concanavalin A (26) were added to all samples. Radioactively labeled phenyl thiohydantoin amino acids were identified by cochromatography with unlabeled standards on polyamide thin-layer sheets (27). The appropriate regions were cut out and added directly to 10 ml of Aquasol (New England Nuclear) for scintillation counting.

RESULTS

Detergent Solubilized H-2 Antigens. The molecular weights of H-2 antigen subunits were determined under dissociating conditions after purification by immune precipitation from NP 40 extracts. $NaDodSO_4$ gel electrophoresis of immune precipitates in the absence of reducing agents showed two species with M_r of 92,000 and 12,000 (Fig. 1A). The smaller component has been previously identified as β_2-microglobulin (7). Elution of the 92,000-dalton component from gels followed by reduction and re-electrophoresis gave only one component with M_r 46,000 (Fig. 1B). This suggests that the 92,000-dalton component represents a disulfide-linked dimer of the H-2 antigen heavy chain. In accord with this result, reduction of immune precipitates with mercaptoethanol resulted in the disappearance of the heavy chain dimer (M_r 92,000), the appearance of the monomer (M_r 46,000), and no change in the β_2-microglobulin (Fig. 1C). Alkylation of cells with iodoacetamide prior to NP 40 extraction resulted in the appearance of monomer heavy chain in unreduced gels and markedly reduced the amount of heavy chain dimer. This indicates that the disulfide linkage between the two heavy chains is formed during or after detergent extraction.

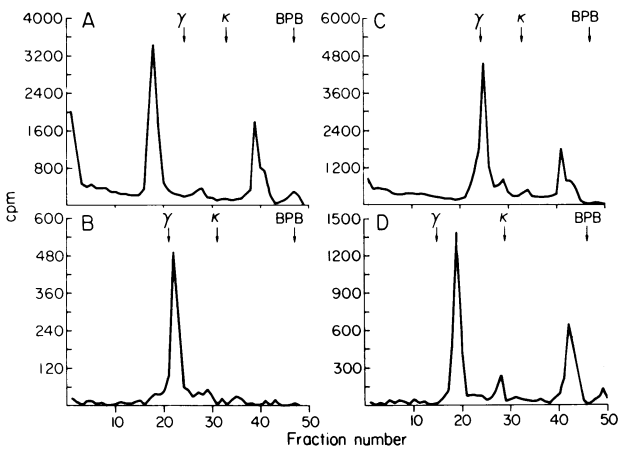

FIG. 1. $NaDodSO_4$-polyacrylamide gel electrophoresis of immune precipitates of NP 40 solubilized H-2K^b antigens from ^{125}I-labeled C57BL/10J spleen cells. (A) Unreduced immune precipitate (10% polyacrylamide gel). (B) Reelectrophoresis of material eluted from fractions 18–20 of the gel shown in panel A after reduction with mercaptoethanol (10% gel). (C) Immune precipitate after reduction with mercaptoethanol on 10% gel. (D) Immune precipitate from papain-treated NP 40 extract after reduction with mercaptoethanol (12% gel). γ, MOPC 21 heavy chain; κ, MOPC 21 light chain; BPB, bromphenol blue.

Identical results were obtained with the K and D gene products of H-2^b, H-2^d, and H-2^k haplotypes, and with either lymphoma or spleen cells. In many cases, however, components other than H-2 antigens were found in the immune precipitates and were identified as C-type viral antigens that had reacted with antiviral antibodies present in the alloantisera (manuscript in preparation).

Molecular weight determinations were also made under other conditions. Gel exclusion chromatography of NP 40 or DOC-solubilized spleen or lymphoma cell membrane material gave a major component with H-2 antigenic activity and M_r 116,000 (Fig. 2A). Lower molecular weight material retaining antigenic activity was also observed frequently. Omission of DOC from the elution buffer or the use of NP 40 instead of DOC increased the apparent M_r considerably, indicating the formation of aggregates. An M_r of 116,000 was the lowest value reproducibly found under conditions appropriate for maintaining antigenic activity. This value is consistent with the hypothesis that intact H-2 antigens in detergent solution are composed of two heavy chains of 46,000

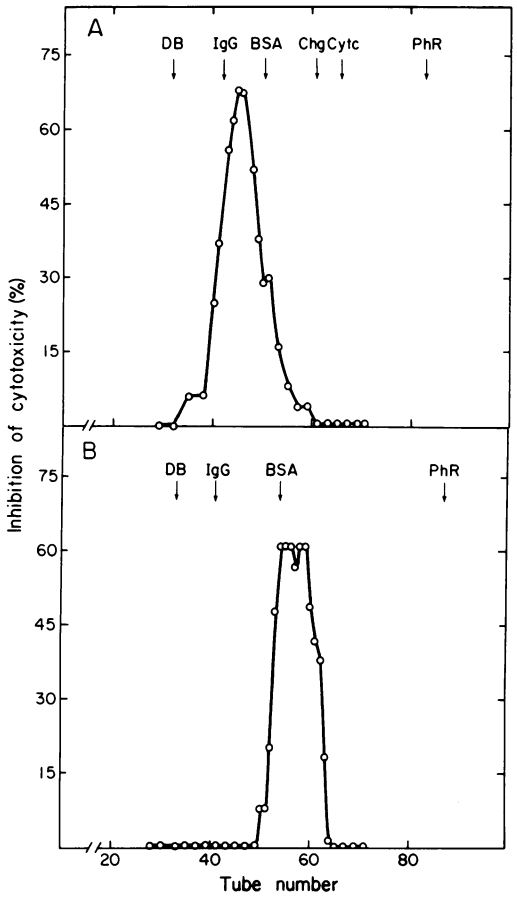

FIG. 2. Gel exclusion chromatography of detergent and papain solubilized H-2 antigens on Sephadex G-200. (A) 1 ml of 0.5% DOC extract of a membrane fraction of C57BL/10J spleens was loaded on a calibrated Sephadex G-200 column (1.2 × 75 cm). Eluant: 0.01 M Tris·HCl buffer, pH 8.0, 0.15 M NaCl, 0.5% DOC, 5 ml/hr. Assay: anti-H-2K^b on ^{51}Cr-loaded C57BL/10J spleen lymphocytes. (B) 1 ml of a 0.5% NP 40 extract of a membrane fraction of 10 BALB/c spleens was digested with papain (see *Materials and Methods*) and loaded on a calibrated Sephadex G-200 column (1.2 × 75 cm). Eluant: 0.01 M Tris·HCl, pH 8.0, 4 ml/hr. Assay: anti-H-2D^d on ^{51}Cr-loaded BALB/c spleen lymphocytes. DB, dextran blue; IgG, MOPC 21 immunoglobulin; BSA, bovine serum albumin; Chg, chymotrypsinogen; Cytc, cytochrome *c*; PhR, phenol red.

daltons each and two noncovalently associated β_2-microglobulin molecules.

Ultracentrifugation of detergent solubilized H-2 antigens on sucrose density gradients in 0.5% DOC gave a peak of antigenic activity at 5.9 S with some minor components at lower S values (Fig. 3). In the absence of DOC most of the H-2 antigenic material was found at the bottom of the centrifuge tube, indicating aggregation of the antigen under these conditions.

Fragments Obtained by Papain Cleavage. NaDodSO$_4$ gel electrophoresis of H-2 antigens obtained by papain treatment of cells or detergent extracts showed components of M_r 39,000 and 12,000, both in the presence and in the absence of reducing agents (Fig. 1D). Occasionally, a minor component of M_r 20,000 was observed. Under nondissociating conditions and in the absence of detergent, gel chromatography of papain solubilized H-2 antigenic material indicated an M_r of approximately 50,000 (Fig. 2B). In sucrose density gradient centrifugation experiments, papain treated detergent extracts showed a major peak of activity at 3.7 S.

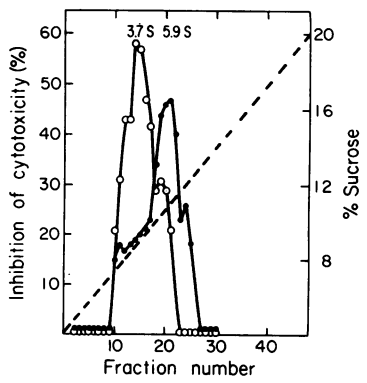

FIG. 3. Sucrose density gradient centrifugation of detergent and papain solubilized H-2 antigens. A 0.5% NP 40 extract (75 μl) from membranes of DBA/2J spleens (10 spleens per ml) was treated with papain, mixed with 75 μl of an untreated extract from C57BL/10J spleen cell membranes, loaded onto a 4–20% sucrose gradient in 0.01 M Tris·HCl, pH 8.0, 0.15 M NaCl, 0.5% DOC, and centrifuged for 25 hr at 35,000 rpm (5°) in an SW 39 Beckman rotor. Fractionation was by tube puncture and drop counting, 6 drops/fraction. Each fraction was assayed for H-2K^d and H-2K^b by inhibition of cytotoxicity of anti-H-2K^d and anti-H-2K^b on DBA/2J and C57BL/10J target cells, respectively. (○) H-2K^d: detergent solubilized and papain treated. (●) H-2K^b: detergent solubilized. (- - -) % sucrose.

Therefore, the results obtained from both the gel chromatography and the ultracentrifugation experiments indicate that the main fragment obtained by papain hydrolysis is monomeric and, in accord with the gel electrophoresis data, consists of a 39,000-dalton heavy chain fragment and one noncovalently associated β_2-microglobulin molecule.

The results of these experiments are summarized in Table 1. In addition, diffusion coefficients and the Stokes' radii were calculated for both the detergent and the papain solubilized H-2 antigens (Table 1) using partition coefficients obtained by gel exclusion chromatography on calibrated Sephadex G-200 columns (Fig. 2). Calculation of the molecular weights (22) using the diffusion and the sedimentation coefficients gave 116,000 for the intact molecule in detergent solution and 49,000 for the major fragment produced by papain cleavage (Table 1).

Comparison of Partial H-2 Heavy Chain Sequences. The amino-terminal sequence of the intact H-2K^b heavy chain was compared with that of its papain fragment in

Table 1. Physicochemical properties of murine histocompatibility antigens in solution

	M_r	Stokes' radius* (Å)	Diffusion coeff.*	Sed. coeff.† (S)
H-2 dimer (in DOC)	116,000‡	47	4.5×10^{-7}	5.9
H chain	46,000§	—	—	—
L chain (β_2)	12,000§	—	—	—
Papain fragment (F_S)	49,000‡	31	6.9×10^{-7}	3.7
H chain (F_H)	39,000§	—	—	—
L chain (β_2)	12,000§	—	—	—

* Estimated from gel chromatography (cm^2 sec^{-1}).
† Sedimentation coefficient calculated from sucrose density gradient centrifugation data.
‡ Calculated using the Svedberg equation (22).
§ NaDodSO$_4$-polyacrylamide gel electrophoresis (17).

Table 2. Partial amino-terminal sequences of H-2 heavy chains

		Residue							
		1	2	3	4	5	6	7	8
H-2K^b	H_2N	—	Pro	His	—	Leu	Arg	Tyr	Phe
H-2K^b F_H†	H_2N	—	—	His	—	—	Arg	Tyr	—
H-2K^d	H_2N	Met*	—	His	—	—	Arg	Tyr	Phe*
H-2K^k	H_2N	—	—	His	—	—	Arg	Tyr	—
H-2D^b	H_2N	—	—	—	—	—	—	Tyr	—
H-2D^d	H_2N	Met	Pro*	His	—	Leu	Arg	Tyr	Phe

* Preliminary results.
† Papain fragment of heavy chain.
—, Denotes the absence of all the other amino acids listed for this gene product.

order to define the relationship between the two polypeptides. Different cell preparations were incubated with individual [^{3}H]amino acids. The specifically labeled H-2 antigen heavy chains and their papain fragments were isolated by preparative gel electrophoresis. For the three amino acids tested, no difference was found between the H-2K^b heavy chain and its papain fragment in the first eight positions (Table 2). These results strongly suggest that the papain solubilized H-2 antigens retain the amino-terminal portion of the peptide chain. The enzyme probably releases the antigens from the cell surface by cleavage of peptide bonds near the carboxyl-terminal end.

In addition, amino-terminal sequences from K and D gene products from different haplotypes were compared to determine whether this region of H-2 molecules contributes to the polymorphism of the H-2 system. The amino-terminal sequences of H-2K^b (EL4 cells), H-2K^d (P388 cells), H-2K^k (AKR thymocytes), H-2D^b (EL4 cells), and H-2D^d (P388 cells) heavy chains were identical at all the positions tested (Table 2). In addition to the data shown in Table 2, H-2K^b, H-2D^d, and H-2K^d heavy chains all had an arginyl residue at position 14, H-2D^d had an arginyl residue at position 21, and H-2K^b had tyrosyl residues at positions 22 and 27.

DISCUSSION

The organization of H-2 molecules on the cell surface, the orientation of amino- and carboxyl-terminal regions of their peptide chains relative to the cell surface, and the nature and distribution of the polymorphism within the heavy chains are of significance in determining the biological function of H-2 antigens. We have formulated a working model (Fig. 4) of the H-2 molecule to provide a framework for further experiments in relating structure to function.

The present data indicate that the detergent solubilized H-2 molecule in solution is composed of two disulfide-linked heavy chains and two noncovalently associated light chains (β_2-microglobulin). Alkylation of intact cells prevents the formation of the disulfide linkage. Preliminary experiments show that a disulfide bridge can be formed between heavy chains on the cell surface by treatment of cells with *o*-phenanthroline and $CuSO_4$ under conditions known to catalyze the formation of disulfide bonds (28).

Recent data from other laboratories have suggested that H-2 and HL-A molecules consist of two disulfide-linked heavy chains and two noncovalently associated β_2-microglobulin chains (10, 11). The amount and nature of higher and lower molecular weight species in both systems are not well defined. Higher polymers of HL-A antigens (10) and H-2 antigens (4) are seen in nonionic detergents and probably result from the formation of large micelles by these detergents. Both 120,000- and 60,000-dalton HL-A species have been observed in DOC (29), and the simultaneous presence of H-2 heavy chain dimers and monomers has been seen in the absence of reducing agents (4). In addition, a 50,000-dalton component of HL-A antigens was observed under dissociating conditions in the absence of reducing agents (11).

These findings and our own observations suggest that the H-2 heavy chain may not exist as a covalently bonded dimer on the cell surface. Monomers are occasionally seen on NaDodSO$_4$-polyacrylamide gels without any prior reduction. Treatment of cells or membrane fractions with iodacetamide prior to detergent extraction greatly increased the amount of heavy chain monomer on NaDodSO$_4$ gels in the absence of reducing agents while oxidation of sulfhydryl groups on cells with *o*-phenanthroline/$CuSO_4$ prior to the described alkylation procedure increased the amount of heavy chain dimer. These findings suggest that the H-2 molecule may exist, at least transiently, in two forms on the cell surface, as a monomer and as a disulfide-linked dimer.

Papain treatment of both cell surfaces and detergent extracts yielded a monomeric fragment (F_H plus L) (Fig. 4), which we have designated F_s for water-soluble fragment to contrast it with the portion of the chain, F_m, that putatively

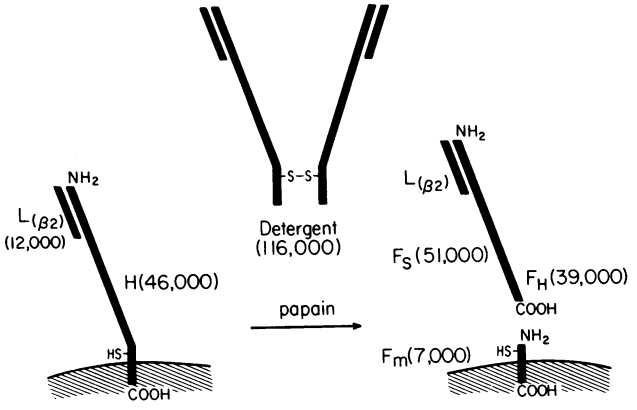

FIG. 4. Molecular anatomy and surface orientation of H-2 antigens. Solubilization of H-2 antigens with detergents gives a structure containing two heavy chains and two light chains with at least one disulfide bond linking the two heavy chains. Papain treatment of cell surfaces or detergent extracts results in fragments of identical size. H, H-2 heavy chain; $L_{(\beta 2)}$, H-2 light chain (β_2-microglobulin); F_s, water-soluble fragment (F_H plus L) obtained after papain treatment of cell surfaces or detergent extracts; F_H, fragment of the H-2 heavy chain obtained after papain treatment; F_m, portion of the H-2 heavy chain cleaved from the bulk of the molecule by papain and apparently associated with the cell membrane. Molecular weights are given in parentheses.

extends into the membrane. This indicates that the noncovalent forces between the H-2 heavy and light chains are located between the F_H fragment and the β_2-microglobulin. We have observed no F_s dimers after papain treatment regardless of the ionic strength. This agrees with results of Peterson *et al.* (11), who found only a 50,000 M_r fragment after papain treatment of HL-A or H-2 antigens, and it is in contrast to observations of Strominger *et al.* (10) who found a noncovalently associated HL-A dimer (M_r 96,000) after papain treatment.

The amino-acid sequences of the intact heavy chain and the F_H fragment of H-2K^b antigens (Table 2), as determined using ^{3}H-labeled antigens, are identical in the three positions tested. These results suggest that the F_H fragment contains the amino-terminal part of the heavy chain and that the F_m fragment is derived from the carboxyl-terminal part of the polypeptide chain. Inasmuch as F_s can be obtained by direct papain treatment of the cell membrane, the amino-terminal end of the H-2 heavy chain probably extends away from the cell surface (Fig. 4) with the carboxyl-terminal region (F_m) associated with the plasma membrane. This orientation is similar to that reported for other cell surface glycoproteins such as glycophorin and membrane-bound immunoglobulin (30–32).

The location and the distribution of those residues that reflect the polymorphism of H-2 antigens probably bear an important relationship to the function of these molecules. The amino-acid sequences for the first eight positions of H-2 gene products from three independent haplotypes (Table 2) are constant for the amino acids tested. These data, however, do not preclude the possibility that other as yet unknown residues in this region express the antigenic polymorphism. Alternatively, these sites may reside in other regions or be distributed over the molecule. The similarity in the sequences of the K and D gene products is consistent with the hypothesis (33) that these genes evolved by duplication from a common ancestral gene. Our present data are too limited to allow any conclusion about the postulated common origin (34, 35) of histocompatibility antigens and immunoglobulins. A more rigorous test of this relationship as well as the identification of sites recognized by cytotoxic T lymphocytes would provide an important basis for establishing the biological function of histocompatibility antigens.

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