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An Improved Preparation of Murine Histocompatibility Antigens (H-2^b) and a Novel Membrane Binding Form of H-$2K^b$

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SUMMARY: A modified preparation of H-2^b is described which results in a greater overall yield, higher proportion of H-$2D^b$ and better preservation of the heavy chain - β_2 microglobulin complex than were obtained previously (9). In the absence of inhibitors of proteolysis, a novel low molecular weight H-$2K^b$ heavy chain is obtained. Results indicate that a portion of the H-$2K^b$ has lost a 3000 dalton peptide from the carboxyl terminus not including the hydrophobic membrane binding peptide.

The major histocompatibiltiy complex (MHC) of mammals codes for a variety of cell surface glycoproteins involved in the immune response (1). The most thoroughly studied of these proteins have been the transplantation antigens designated HLA in man and H-2 in the mouse. HLA consists of at least three different proteins coded for by separate loci designated HLA-A, -B and -C. H-2 also consists of at least three molecules coded for by separate loci designated H-2K, D and L. By using alloantisera and metabolically labeled cells it has been established that H-2 proteins and HLA proteins coded for by any of the three loci have the same basic overall structure. They consist of a heavy chain having a molecular weight of about 50,000, which bears the serological specificities, non-covalently linked to a 12,000 dalton protein which is β_2 microglobulin (2).

The large scale purification of all of these molecules has been reported by several groups (3-11). The molecules from mouse and man have similar amino acid sequences (13) and virtually identical chemical structure. The heavy chain can be cleaved by papain into an amino-terminal hydrophobic segment (p-H-2^b) which normally extends away from the membrane and contains the allo-

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antigenic specificities (5,7,8) and a hydrophobic segment which anchors the protein to the plasma membrane (4,9, unpublished data of M.J. Rogers). The hydrophobic segment has a molecular weight of about 6,000 in the case of H-2^b (9) and about 10,000 in the case of HLA (4). The 10,000 dalton carboxyl terminal fragment of HLA could be further subdivided into a 5,000 dalton hydrophobic region responsible for binding to the membrane and an ultimate carboxy terminal, 5000 dalton, hydrophilic segment which normally extends into the cytoplasm of the cell (6). This subdivision of the hydrophobic segment has not been reported for H-2.

In this paper a modified method for the purification of H-2^b is reported which results in a revised estimate for the ratio of H-2K^b to H-2D^b. Moreover, in the absence of proteolytic enzyme inhibitors a species of H-2K^b is purified which can bind to membranes and is intermediate in molecular weight between H-2^b and p<u>H</u>-2^b.

MATERIALS AND METHODS: RBL-5 tumor cells were maintained in ascites in C57B/6 mice, harvested, washed twice with phosphate buffered saline and frozen as a packed pellet at -70°C.

Broad reacting anti H-2^b antisera was prepared by skin grafting C3H mice with B/6 skins and hyperimmunizing with B/6 spleen cells. Anti H-2K^b directed against the private specificity H-2.33 (B10.D2 XA F_1 anti B10.A 5R) and anti H-2D^b directed against the private specificity H-2.2 (B10.A 5R X LP.RIII F_1 anti B10) were obtained from the Research Resources Branch, NIAID, NIH.

Other materials used in this work have been described previously (9).

During the purification of H-2^b, antigen was monitored by the radioimmunoassay described previously (9). This assay employs broad reacting C3H anti B/6 alloantisera and ^{125}I-p<u>H</u>-2^b. Since the p<u>H</u>-2^b preparation contains only the H-2K^b molecule (see text) the H-2D^b molecule is not detected in this assay. β_2 microglobulin was also determined by radioimmunoassay as described previously (9).

The purification of H-2^b was a modification of the procedure reported previously (9) in that whole cells instead of purified membranes were extracted with detergent and gel filtration was performed at the end rather than the beginning of the procedure. RBL-5 cells were thawed and suspended in 10 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl, 1 mM dithiothreitol 10μg per ml of aprotinin, 10 μg per ml of L-1-tosylamide-2-phenylethyl chloromethyl ketone, and 100 μg per ml of α toluene sulfonyl fluoride (all three proteolytic enzyme inhibitors from Sigma Chemical Co., St. Louis, MO). The suspension was brought to 0.7% Triton X100 and cells were extracted for 40 min at 4° and then centrifuged at 100,000 xg for 45 min. The supernatant was applied directly to a 45 ml <u>Lens culinaris</u> lectin affinity column equilibrated as described previously (9, except 0.5% Triton X100 was used instead of deoxycholate) and eluted with 3% α-methyl mannoside. After dialysis, the H-2^b was applied to and eluted from successive immunoadsorbants of normal sheep gamma globulin and sheep anti-H-2^b gamma globulin presicely as described previously (9) except that the anti H-2^b immunoadsorbant was poured directly

onto 170 ml of Sephadex G-25 which removed NaSCN immediately after H-2^b emerged from the affinity column. The detergent in the preparation was then changed from 0.5% Triton X100 to 0.2% deoxycholate by affinity chromatography as described (9) and the H-2^b was concentrated to 2 ml by ultrafiltration and lyophilization. The preparation, now in 10 mM Tris pH 8.3, 1-2% deoxycholate, 1 µg per ml aprotinin, 0.02% NaN_3 by virtue of the concentration procedure, was brought to 2 mM DTT and applied to a 1 x 130 cm Ultrogel AcA34 column equilibrated as described previously. Pure H-2^b, which eluted as a single band with an apparent molecular weight of 90,000 was dialyzed into 0.1% deoxycholate concentrated to 1-2 mg per ml by lyophilization, subjected to gel filtration on a 90 ml Sephadex G-25 column to remove detergent, and stored at -70°.

Iodination, immunoprecipitation and SDS polyacrylamide gel electrophoresis (SDS-PAGE) of purified H-2^b were performed by published procedures (9). Papain digestion of [^{125}I]H-2^b was performed at a protein:papain ratio of 100 (w:w) as described previously (9).

H-2^b was incorporated into artificial lipid vesicles by a slight modification of the detergent dialysis procedure of Engelhard et al. (12). The vesicles were brought to 50% (w:w) with solid sucrose, overlayed with 40% sucrose (w:v) followed with buffer, and centrifuged at 150,000 xg overnight in a swinging bucket rotor. Trace amounts of (^{14}C) phosphatidylcholine were included as a marker in the egg lecithin.

RESULTS: Table I summarizes the results of a typical purification of H-2^b. The overall purification is similar to that obtained earlier with purified plasma membranes as the starting material (1159 fold here vs. 1525 fold previously, ref. 9) with three exceptions. 1) The yield in this preparation, is nearly two-fold greater, largely because the 70% loss of H-2^b during plasma membrane isolation was avoided (9). This is the best yield of a mixture of H-2K^b and H-2D^b reported to date. 2) The ratio of H-2K^b to H-2D^b determined by immunoprecipitation of [^{125}I]H-2^b with monospecific alloantisera was 1.4:1. In the previous preparation the ratio was 5:1. 3) The immediate removal of NaSCN from the H-2^b eluted from the immunoabsorbant by gel filtration resulted in 70% to 80% of the H-2^b- β_2 microglobulin complex remaining intact as estimated by radioimmunoassay of β_2 microglobulin (data not shown). In the previous preparation, only 50% of the complex survived treatment with NaSCN.

The presence of proteolytic enzyme inhibitors during the preparation was essential, especially during the initial solublization. In the presence of inhibitors a single peak of H-2^b eluted from the final gel filtration column with an apparent molecular weight of 90,000 as observed previously (9). The material in this peak appeared as two bands on SDS-PAGE with molecular weights of 50,000 and 12,000 with only minor impurities (Fig. 1, lane 2). In the

TABLE I

Summary of the Purification of dH-2^b from RBL-5 Tumor Cells

Fraction	Protein mg	H-2^b units x 10^{-5}	Specific Activity units/mg x 10^{-2}	Purification - fold	Yield %
Cells	10800[a]	7.7[d]	0.7	1	100
Triton extract	6510	7.8	1.2	1.7	100
Lectin	90	5.8	64.9	91.2	76
Immunoadsorbant	8.8	2.8	313.4	440.1	36
AcA34[b]	1.7	1.4	811.0	1158.6	18
AcA34-I[c]	2.0	0.5	250.0	357.1	4
AcA34-II[c]	1.8	1.0	560.0	800.0	11

[a] This amount of protein is obtained from 7.5 x 10^{10} cells

[b] Gel filtration data for preparation performed in the presence of proteolytic enzyme inhibitors

[c] Gel filtration data for an identical preparation performed without using proteolytic inhibitors at any step.

[d] Activity determined by radioimmunoassay

absence of inhibitors, two peaks of H-2^b eluted from the gel filtration column with apparent molecular weights of 90,000 (peak I) and 50,000 (peak II). The H-2^b from both of these peaks was less pure than the H-2^b obtained in the

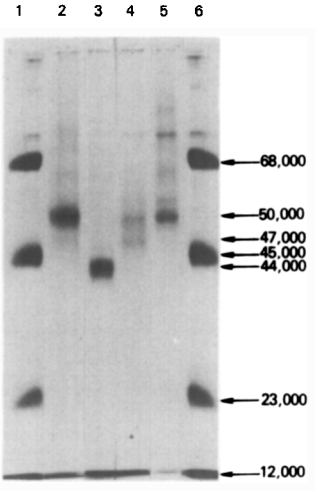

Fig. 1. SDS polyacrylamide gel electrophoresis of purified H-2^b. Electrophoresis was performed on 10 µg samples of protein as described in Materials and Methods and stained with coomassie blue. Lane 1: standard proteins bovine serum albumin (68,000 daltons), ovalbumin (45,000 daltons), mouse immunoglobulin light chain (23,000 daltons) and cytochrome c (12,000 daltons). Lane 2: H-2^b purified from RBL-5 cells as described in Materials and Methods. Lane 3: pH-2^b purified from papain extracts of B/6 livers as described previously (7). Lane 4: H-2^b from preparation without inhibitors of proteolysis peak II. Lane 5: H-2^b from prepration without inhbitors of proteolysis peak I. Lane 6: standard proteins as in lane 1.

presence of proteolytic enzyme inhibitors (Table I). Peak I material resolved into 5 major bands besides β_2 microglobulin on SDS-PAGE, the most prominent corresponding in molecular weight to pure H-2^b (compare lanes 2 and 5, Fig. 1). Besides β_2 microglobulin the most prominent peak II bands had apparent molecular weights of 50,000 (corresponding to H-2^b) and 47,000 (Fig. 1, lane 4).

In order to identify the species of H-2^b present in peaks I and II, protein from each peak was labeled with ^{125}I and immunoprecipitated with monospecific alloantisera (Fig. 2). The peak I H-2^b contained almost exclusively H-2K^b with an apparent molecular weight of 50,000. Peak II H-2^b contained H-2D^b with an apparent molecular weight of 50,000 and H-2K^b with an apparent molecular weight of 47,000. Immunoprecipitation of [^{125}I]H-2^b prepared in the presence of inhibitors of proteolysis showed that both H-2D^b and H-2K^b had an apparent molecular weight of 50,000 on SDS-PAGE (data not shown). Therefore, the molecular weight difference observed on SDS-PAGE for H-2K^b from peaks I and II is not caused by migration artifacts due to carbohydrate. These results strongly suggest that when not protected by inhibitors, a 3000 dalton peptide is cleaved from a portion of the H-2K^b during purification to produce a stable, 47,000 dalton species.

Fig. 2 also shows the results of papain digestion of peak I and peak II H-2^b. The two forms of H-2K^b in peaks I and II were both reduced in molecular weight to 44,000 as expected for pH-2^b (see Fig. 1, lane 3). The H-2D^b from peak II as well as very small amounts of H-2D^b in peak I was apparently very sensitive to papain because none could be immunoprecipitated after digestion.

The fact that the 47,000 dalton H-2K^b from peak II was reduced to a species having all the properties of the amino-terminal cleavage fragment, pH-2^b (i.e., molecular weight of 44,000, expresses alloantigen, binds β_2 microglobulin) when treated with papain indicated that the 3000 dalton fragment missing from H-2K^b had been removed from the carboxyl terminus. Since the membrane binding segment of H-2^b is also located at the carboxyl terminus,

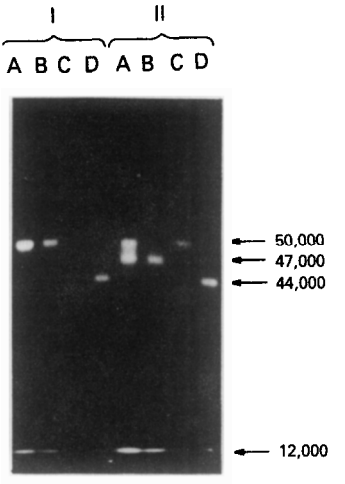

Fig. 2. SDS polyacrylamide gel electrophoresis of immunoprecipitates of H-2^b from peaks I and II with and without papain digestion. H-2^b from fractions I (left) and II were radiolabeled with ^{125}I and immunoprecipitated directly (lanes A, B and C) or subjected to papain digestion as described in Materials and Methods (lane D). Antisera used were: A, C3H anti B/6, B and D, monospecific anti H-$2K^b$; C, monospecific anti H-$2D^b$.

the ability of the 47,000 dalton fragment of H-$2K^b$ to bind to membrane was assessed. Fig. 3 shows the results of an experiment in which H-2^b from peak I and peak II was incorporated into lipid vesicles and separated from unbound proteins on a sucrose gradient. Since H-2^b was monitored by the radioimmunoassay only H-$2K^b$ was detected in the experiment shown. The data clearly show

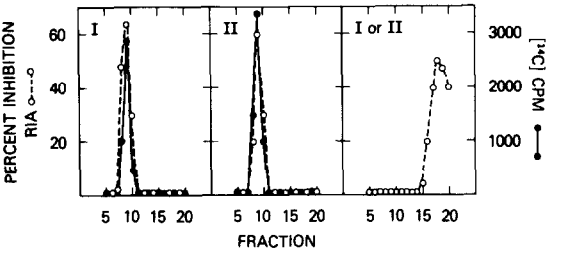

Fig. 3. The binding of H-2^b from Fraction I and II to artificial membranes prepared from egg lecithin. H-2^b from Fraction I (left) or Fraction II (middle) was incorporated into liposomes as described and floated on a discontinuous gradient. Fractions were collected and assayed for (•-•) egg lecithin by liquid scintillation counting or (o--o) H-2^b by radioimmunoassay. The figure on the right is the profile obtained for a gradient containing H-2^b from either Fraction I or II that had been subjected to the reconstitution procedure in the absence of egg lecithin. In each case the top of the gradient is on the left and the buffer-40% sucrose interface is at fraction 8.

that both 50,000 dalton H-2K^b from peak I and 47,000 dalton H-2K^b from peak II are completely associated with lipid under these reconstitution conditions. When pH-2^b was subjected to the reconstitution procedure, no protein was associated with the artificial membranes, demonstrating that the 6000 dalton carboxyl terminal peptide is essential for binding to membrane and that no trapping of protein within vesicles occurs under these reconstitution conditions (data not shown).

DISCUSSION: The results reported herein describe an improved method for the purification of H-2^b which results in a very high yield of pure product (18%) and approximately equal amounts of H-2D^b and H-2K^b. An earlier preparation from this laboratory contained a 5-fold excess of H-2K^b over H-2D^b. Thus, this new evidence indicates that the two H-2^b gene products are expressed on the RBL-5 tumor cell in approximately equal amounts.

In the absence of inhibitors of proteolysis, relatively impure H-2^b was obtained (Table I). However, a new, previously unreported species of H-2K^b was produced which appears to have lost a 3000 dalton peptide from its carboxyl terminus. Although an amino terminal sequence determination is necessary to prove this unequivocally, the fact that a normal 44,000 dalton pH-2K^b was generated from the 47,000 dalton species by papain digestion (Fig. 2) provides strong evidence that the amino terminus is identical on 50,000 dalton H-2K^b and 47,000 dalton H-2Kb.

In spite of the loss of this 3000 dalton carboxyl terminal peptide, H-2K^b was still bound to artificial membranes (Fig. 3). Since the 44,000 dalton papain fragment of H-2^b does not bind to membranes the hydrophobic, membrane binding segment of H-2K^b must be intact in the 47,000 dalton species. Thus, these data indicate that the 6000 dalton carboxyl terminal of H-2K^b which is cleaved by papain can be subdivided into a 3000 dalton penultimate membrane binding segment and an ultimate 3000 dalton peptide not necessary for membrane binding. Except for the molecular weights of these peptides which are 40% lower, this is exactly analogous to the situation in HLA (6). Whether the

carboxyl terminal of H-2^b is hydrophilic, exposed within the cell, and phosphorylated as has been shown for HLA (6) remains to be seen.

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