

## STRUCTURE OF MURINE Ia ANTIGENS

### Two-Dimensional Electrophoretic Analyses and High-Pressure Liquid Chromatography Tryptic Peptide Maps of Products of the *I-A* and *I-E* Subregions and of an Associated Invariant Polypeptide\*

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The *I* region of the major histocompatibility complex (*H-2*) of the mouse controls a variety of immunologically related phenomena, including immune responsiveness and cellular interactions among lymphocytes and antigen-presenting cells (1–4). The only gene products from the *I* region that have been identified by immunochemical techniques are the Ia (*I* region-associated) antigens (5). Evidence involving the inhibitory effects of anti-Ia antibodies suggests that these Ia antigens may mediate at least some of the immunological phenomena controlled by the genes of the *I* region (6, 7).

The *I* region is genetically complex and can be divided into at least five subregions, *I-A*, *I-B*, *I-J*, *I-E*, and *I-C*, by recombinational analysis (8). Loci that map to the *I-A* and *I-E* subregions have been shown to encode the Ia antigens, which appear to be highly polymorphic by serological analysis and are expressed predominantly on B lymphocytes (9, 10). The Ia molecules are integral cell surface glycoproteins and consist of two distinct, noncovalently associated polypeptide chains:  $A_\alpha$  and  $A_\beta$  compose the Ia A molecules immunoprecipitated using anti-A subregion sera, whereas  $E_\alpha$  and  $E_\beta$  constitute the Ia E molecules isolated using anti-E subregion reagents. The larger polypeptides,  $A_\alpha$  and  $E_\alpha$ , have apparent molecular weights of 33,000–35,000, whereas the smaller polypeptides,  $A_\beta$  and  $E_\beta$ , have apparent molecular weights of 27,000–29,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).<sup>1</sup>

These Ia polypeptides have been partially characterized by us and by others (11–

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<sup>1</sup> Abbreviations used in this paper: HPLC, high-pressure liquid chromatography; IEF, isoelectric focusing; 1D or 2D SDS-PAGE, one-dimensional or two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SAC, *Staphylococcus aureus* Cowan I strain; TCA, trichloroacetic acid; TPCK, L(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone.

15). Using the techniques of partial N-terminal, amino acid sequencing and high-pressure liquid chromatography (HPLC) tryptic peptide mapping, we have shown that the  $E_\alpha$  polypeptides have striking homology with human p34 chains, at least at their N-termini; that the  $E_\alpha^d$  and  $E_\alpha^k$  polypeptides are very similar, if not identical; that the  $A_\alpha$ ,  $A_\beta$ , and  $E_\beta$  polypeptides of various haplotypes differ by multiple amino acid substitutions; and that the genes encoding these proteins map to the *H-2* complex (16-20).

Two-dimensional electrophoretic analyses of Ia immunoprecipitates by Jones et al. (21) have shown that a third protein, designated the invariant polypeptide or  $I_i$ , which is basic in character and appears to be nonpolymorphic, specifically co-immunoprecipitates with Ia molecules.<sup>2</sup> McKean and his coworkers (22) have recently demonstrated that the interaction of this protein with Ia molecules is not an artifact of the isolation procedures. Proteins that appear to be the counterparts of  $I_i$  have been recognized in man and the rat (23, 24).

In this paper, we investigate the relation of this invariant protein to the polypeptides that we have previously partially characterized and we extend our genetic mapping studies of the Ia polypeptides. We show that  $I_i$  does co-immunoprecipitate with our Ia molecules, that the strength of its interaction with Ia polypeptides varies with haplotype, and that it is not a precursor of the  $A_\alpha$ ,  $A_\beta$ ,  $E_\alpha$ , or  $E_\beta$  polypeptides. We also demonstrate that the polypeptides we have previously characterized are contaminated with very little, if any, invariant protein. Further, we have used our HPCL tryptic peptide map technique to formally map the genes encoding the  $A_\alpha$ ,  $A_\beta$ , and  $E_\beta$  polypeptides to the *I-A* subregion using recombinant and  $F_1$  hybrid mice.

### Materials and Methods

*Mice.* All mice used were obtained from the mouse colonies of Drs. McDevitt and Frelinger.

*Antisera.* Monoclonal anti- $A^k$  antibodies were derived from the hybridoma cell line 10-2.16 (The Salk Institute, San Diego, Calif.). The following alloantisera were used: A.TL anti-A.TH ( $A^k$ ), C3H anti-C.SW ( $A^b$ ), and B10XHTI $F_1$  anti-B10.A(3R) ( $E^k$ ). Their specificities have been discussed previously (16, 17, 19).

#### *Isolation of Ia Antigens*

**RADIOLABELING.** The procedures for radiolabeling and isolating Ia antigens have been described in detail elsewhere (16). Briefly, splenic lymphocytes at a concentration of  $5 \times 10^7$  cells/ml (including erythrocytes) were cultured for 5-9 h in Hanks' balanced salt solution with 10 mM Hepes, 5% dialyzed fetal calf serum, and radiolabeled amino acids (New England Nuclear, Boston, Mass.). The cells were lysed at the same concentration with TST buffer (0.01 M Tris, 0.15 M NaCl, pH 7.3, and 0.5% Triton X-100) and, after centrifugation, the supernate (cell lysate) was stored at  $-70^\circ\text{C}$ .

**IMMUNOPRECIPITATION.** Antigen-antibody complexes were isolated using formalin-fixed *Staphylococcus aureus* Cowan I strain (SAC) as described by Cullen and Schwartz (25). The radiolabeled Ia antigens were eluted from the SAC by boiling for 2 min in "elution" buffer (0.063 M Tris, pH 6.8, 2% 2-mercaptoethanol, and 2% SDS). In studies involving immunoprecipitation in the presence of 0.5% SDS, the washing buffer was TST buffer containing 0.1% SDS.

**ONE-DIMENSIONAL (1D)-SDS-PAGE.** Radiolabeled Ia polypeptides in elution buffer (100-300  $\mu\text{l}$ ) were separated on 10% polyacrylamide gels (26) and were eluted from 1-mm gel slices using 0.01% SDS. In dual-label studies,  $^3\text{H}$  and  $^{35}\text{S}$  dpm were calculated directly by a Beckmann LS9000 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.), using experimentally determined quench curves.

<sup>2</sup> This polypeptide has been alternatively designated  $A_e$  (40) because it is encoded by a gene located within the *I-A* subregion.

**HPLC Tryptic Peptide Mapping.** The procedure used is essentially that described by McMillan et al. (18). Each polypeptide was mixed with 0.25 mg porcine immunoglobulin and lyophilized. The protein was then reduced, alkylated, and digested with L-(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone (TPCK)-trypsin (Worthington Biochemical Corp., Freehold, N. J.), and the resulting tryptic peptides were analyzed by HPLC using either a 25-cm Zorbax-CN column (DuPont Instruments, Wilmington, Del.; Figs. 5-7 and 9) or a 25-cm Zorbax-ODS column (Fig. 8) at 49°C, and a phosphate/acetone solvent system. 0.5-ml fractions were collected every 30 s.

#### *Two-Dimensional (2D) Electrophoresis*

The samples were loaded at the anode and separated in the first dimension by isoelectric focusing (IEF) and in the second dimension by SDS-PAGE. This is a modification of the methods described by O'Farrell (27, 28).

**FIRST DIMENSION.** The IEF gel mixture was made as follows: 0.8 ml twice-distilled water, 1.0 ml 10% (vol/vol) Triton X-100 solution, 0.84 ml stock acrylamide solution (100 ml contained 28.38 g acrylamide, 1.62 g bisacrylamide [Bio-Rad Laboratories, Richmond, Calif.]) and LKB ampholines (200  $\mu$ l, pH 3.5-10; 70  $\mu$ l, pH 9-11; 70  $\mu$ l, pH 2.5-4) were added to 2.75 g urea. 8.5  $\mu$ l ammonium persulfate and 6.3  $\mu$ l N,N,N',N'-tetramethylethylenediamine were used for polymerization. The solution was overlaid using 8 M urea and "lysis" buffer (9.5 M urea, 2% Triton X-100, 1.6% ampholines, pH 5-7, 0.4% ampholines, pH 3.5-10, and 5% 2-mercaptoethanol). After 2 h, the overlay solution was removed and, without 'prefocusing' each sample (in lysis buffer), was loaded at the anode and overlaid with 10  $\mu$ l of a solution containing 8 M urea, ampholines (0.2% pH 3.5-10, 0.8% pH 5-7), and 0.5 mg/ml each glutamic and aspartic acids. The samples were subjected to IEF at 500 V for 10-12 h. The gels to be analyzed in a second dimension were extruded into a solution containing 0.18 M Tris, pH 6.8, 30% glycerol, 7% SDS, and 12.5% 2-mercaptoethanol.

**SECOND DIMENSION.** The IEF gels were sealed on top of the stack of an SDS slab gel (10% polyacrylamide) and electrophoresed as described by O'Farrell (27). The gels were prepared for fluorography using the method of Bonner and Laskey (29) and were then dried and exposed to reflashed Kodak XR-5 film (Eastman Kodak Co., Rochester, N. Y.; 30).

**METHIONINE PEPTIDE MAPS OF THE INVARIANT POLYPEPTIDE.** The A<sup>s</sup> and E<sup>k</sup> molecules were sequentially immunoprecipitated from a [<sup>35</sup>S]methionine-labeled B10.HTT cell lysate and run on 1D-SDS-PAGE. The proteins of ~31,000 mol wt (intermediate between  $\alpha$  and  $\beta$ ) were mixed with carrier, reduced, alkylated, and precipitated with trichloroacetic acid (TCA). The precipitates were dissolved in lysis buffer and subjected to IEF. The gels were sliced into 2-mm discs and incubated in 0.5 ml containing 0.05 M ammonium bicarbonate and 0.05% SDS. 10% aliquots were counted, and the remaining 90% of the relevant fractions were mixed with 0.25 mg porcine gammaglobulin that had been previously reduced and alkylated. After TCA precipitation, the proteins were digested with trypsin, and the peptides were mapped using HPLC.

## Results

**1D-SDS-PAGE Analyses of <sup>35</sup>S- and <sup>3</sup>H-labeled Ia Immunoprecipitates.** In our characterizations of Ia polypeptides by partial amino acid analyses and HPLC tryptic peptide mapping, we had previously used tritiated amino acids. In contrast, in the 2D electrophoresis studies by Jones (31) and others (32), [<sup>35</sup>S]methionine was often used to facilitate analysis by autoradiography. To compare both types of incorporation, we labeled B10.A(1R) and B10.HTT spleen lymphocytes with [<sup>3</sup>H]phenylalanine and [<sup>35</sup>S]methionine. Conventional immunoprecipitation in TST buffer using anti-Ia serum, followed by analysis on 1D-SDS-PAGE, gave the profiles shown in Fig. 1, panels i and iii. The A <sub>$\alpha$</sub>  and A <sub>$\beta$</sub>  polypeptides that we have previously isolated are the predominant species in the <sup>3</sup>H-labeled immunoprecipitate, whereas a third species, having an apparent molecular weight intermediate between the  $\alpha$  and  $\beta$  polypeptides,

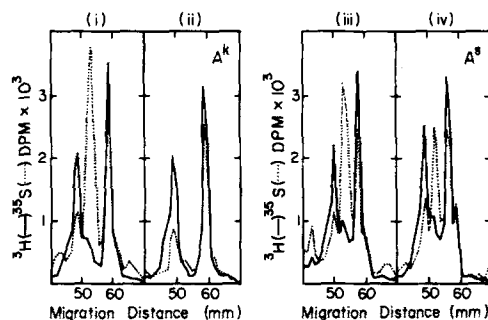


FIG. 1. Portions of SDS-PAGE patterns (10% reducing gels) of  $A^k$  molecules immunoprecipitated from a B10.A(1R) cell lysate (panels i and ii), and  $A^s$  molecules immunoprecipitated from a B10.HTT cell lysate (panels iii and iv). The radioactivity is plotted on the ordinate; (—) represents incorporated [ $^3\text{H}$ ]phenylalanine; (· · ·) denotes [ $^{35}\text{S}$ ]methionine. Migration distance is plotted on the abscissa. The buffer used during the immunoprecipitation procedure was TST in panels i and iii, and TST containing 0.5% SDS in panels ii and iv.

is the dominant species when [ $^{35}\text{S}$ ]methionine is used as radiolabel. We have confirmed that this is the invariant polypeptide, first described by Jones et al. (21), by our 2D gel analyses reported later in this paper. In all our [ $^{35}\text{S}$ ]methionine-labeled Ia immunoprecipitates (both A and E), we find that this polypeptide is always present and always appears to be the predominant species.

To determine whether the binding of this invariant protein to Ia molecules is as strong as the association between the  $\alpha$  and  $\beta$  polypeptides, immunoprecipitations were carried out in the presence of 0.5% SDS. Such conditions have been reported to dissociate actin from transplantation antigens (33). Fig. 1, panel ii shows the analysis of the  $A^k$  molecule in which the invariant polypeptide is clearly absent when the immunoprecipitation is carried out in TST buffer containing 0.5% SDS. Similar results have been obtained by Moosic et al. (22), using pyrrolidinone. Panel iv shows the analysis of the  $A^s$  molecule in the presence of SDS, in which the amount of the invariant polypeptide has been reduced only slightly. We conclude, therefore, that  $I_1$  can be tightly bound at least to some Ia molecules.

**2D Electrophoretic Analyses of Ia Molecules and Isolated Ia Polypeptides.** To determine whether the invariant protein is a contaminant of any of the Ia polypeptides that we have partially sequenced or peptide mapped, we isolated polypeptides from preparative 1D-SDS-PAGE gels by our usual method, submitted them individually to 2D electrophoresis, and compared them to a 2D analysis of the appropriate total Ia immunoprecipitate. Fig. 2 shows the fluorographs of the 2D gels obtained from  $^3\text{H}$ - and  $^{35}\text{S}$ -labeled total immunoprecipitates of the  $A^s$  and  $E^k$  molecules. Small fractions of the immunoprecipitates were also submitted both to analytical SDS-PAGE and IEF, and the distribution of radiolabel was determined by scintillation counting. The familiar radioactivity profiles obtained from 1D-SDS-PAGE gels are illustrated on the right in Fig. 2 and show a  $^{35}\text{S}$ -rich peak,  $I_1$ , lying between the Ia polypeptides. The IEF radioactivity profiles, shown above the appropriate fluorograph, demonstrate that the  $^{35}\text{S}$ -rich polypeptide has a pI of  $\sim 8$ , and is the same for the  $A^s$  and  $E^k$  molecules.

Fig. 3 shows sections of the 2D gels of isolated Ia polypeptide chains. Panel i shows the analysis of  $\sim 1.7\%$  ( $\sim 5 \times 10^3$  cpm/ $\sim 3 \times 10^5$  cpm) of the samples  $E^d_\alpha$  and  $E^d_\beta$ , whose

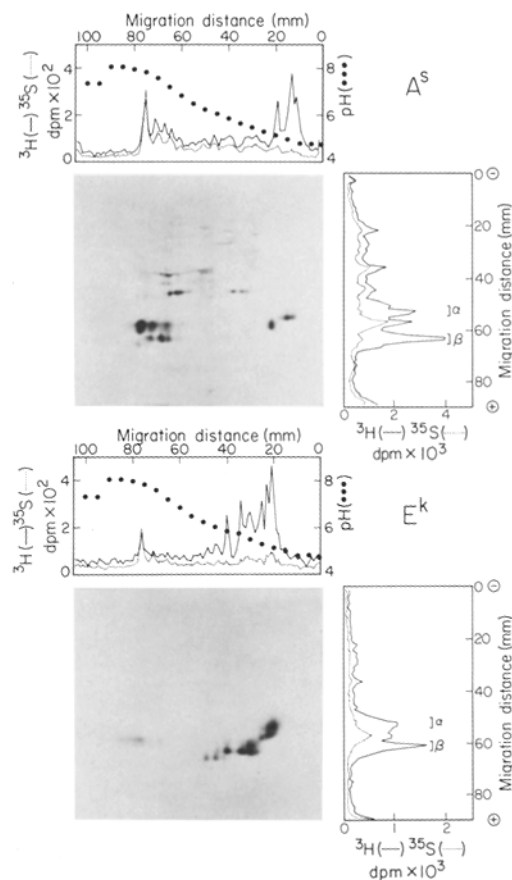


FIG. 2. Fluorographs of 2D gels of  $A^s$  molecules (top) and  $E^k$  molecules (bottom) immunoprecipitated from B10.HTT spleen cell lysates. The SDS-PAGE pattern of the appropriate immunoprecipitate is plotted to the right of each fluorograph and the IEF pattern is shown above each fluorograph. (—) represents incorporated [ $^3\text{H}$ ]phenylalanine and [ $^{35}\text{S}$ ]tyrosine, (••••) indicates [ $^{35}\text{S}$ ]methionine. pH is denoted by (••••).

partial amino acid sequences were reported by McMillan et al. (17). The invariant polypeptide was absent from both samples. Panels ii and iii show the analyses of isolated  $^3\text{H}$ - and  $^{35}\text{S}$ -labeled  $A_\alpha^s$ ,  $A_\beta^s$ ,  $E_\alpha^k$ , and  $E_\beta^k$  polypeptides. They were isolated initially from 1D-SDS-PAGE gels in a manner identical to that used for our HPLC tryptic peptide mapping. Only  $A_\beta^s$  and  $E_\beta^k$  show slight contamination with the invariant polypeptide. Panel iv shows the result of analyzing all the radioactivity of molecular weight intermediate between the Ia polypeptides for both the A and the E immunoprecipitates. A large amount of invariant polypeptide is present in each. Peptide mapping of the acidic polypeptide in the left panel showed that its tryptic peptides eluted in a manner identical to those of the  $A_\alpha^s$  polypeptide, which we have previously characterized (panel ii). Because this polypeptide is more basic and smaller in molecular weight than  $A_\alpha^s$ , we conclude that it is probably an incompletely processed  $\alpha$ -chain lacking some (or all) of its carbohydrate moiety(ies). By analogy, in gel iv, the acidic polypeptide in the right panel is smaller in size and slightly more

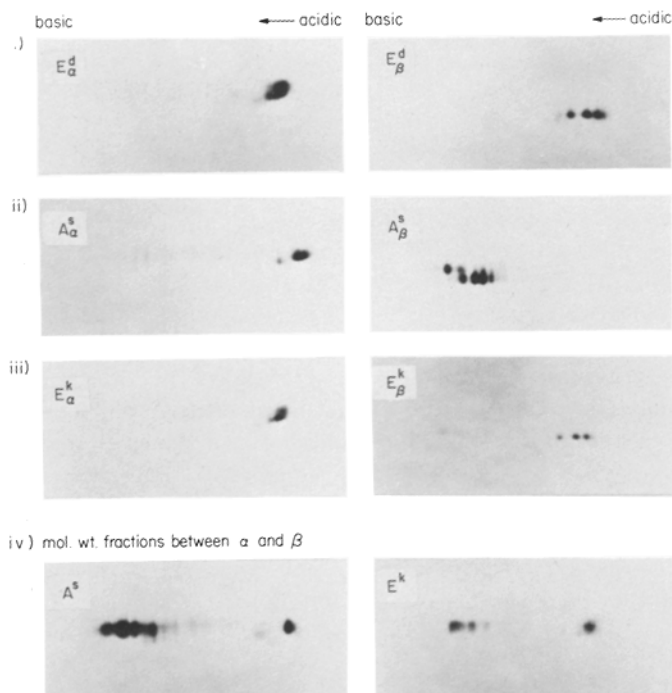


FIG. 3. Portions of fluorographs of 2D gels of Ia polypeptides, previously isolated by immunoprecipitation and SDS-PAGE. The identity of each chain is indicated on the fluorograph. In panel i, the polypeptides were isolated from a B10.D2 cell lysate. [<sup>3</sup>H]alanine, tyrosine, valine, isoleucine, phenylalanine, leucine, and proline were used as radiolabels. The partial N-terminal amino acid sequences of these proteins has been reported in reference 17. In panels ii, iii, and iv, the polypeptides were isolated from a B10.HTT cell lysate. [<sup>3</sup>H]phenylalanine and tyrosine and [<sup>35</sup>S]methionine were used as radiolabels. In panel iv, proteins of molecular weight intermediate between A<sub>α</sub>(E<sub>α</sub>) and A<sub>β</sub>(E<sub>β</sub>) are shown in the left (right) fluorograph.

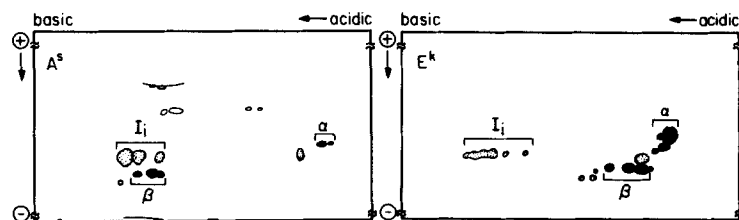


FIG. 4. Diagrammatic representations of portions of the fluorographs in Fig. 2, indicating the various polypeptide chains. Black spots represent proteins for which we have obtained tryptic peptide map and partial N-terminal amino acid sequence data. Stippled spots represent proteins that we have analyzed by tryptic peptide mapping.

basic than the E<sub>α</sub><sup>k</sup> polypeptide (panel iii), which suggests that it is an incomplete form of the E<sub>α</sub><sup>k</sup> polypeptide.

In Fig. 4, the schematic diagrams depict portions of the 2D gels in Fig. 2 and summarize the results shown in Fig. 3.

*Comparative HPLC Tryptic Peptide Mapping on [<sup>3</sup>H]phenylalanine-labeled Polypeptides Isolated by 1D-SDS-PAGE.* Having ascertained that the proteins we have partially chemically characterized contain little or no invariant polypeptide, we have used

HPLC to analyze the radiolabeled tryptic peptides derived from I<sub>a</sub> polypeptides and have genetically mapped the loci encoding the A<sub>α</sub>, A<sub>β</sub>, and E<sub>β</sub> chains. Similar experiments have been reported by other laboratories (34–38) in which tryptic peptides were analyzed using ion-exchange chromatography and IEF.

One of the major advantages of HPLC over conventional ion-exchange chromatography is that its extreme reproducibility enables us to compare many peptide maps run over a period of several weeks. In contrast, only two polypeptides analyzed as a mixture, one <sup>14</sup>C-labeled, the other <sup>3</sup>H-labeled, can be compared by ion-exchange chromatography.

In this study, we have used [<sup>3</sup>H]phenylalanine as the radiolabel for three reasons: (a) phenylalanine can be obtained with high specific activity (often >100 Ci/mM), (b) it is readily biosynthetically incorporated into I<sub>a</sub> polypeptides, and (c) it is a more common amino acid than tyrosine and less common than leucine. In this way, we hoped to observe more tryptic peptides than in our previously reported studies using [<sup>3</sup>H]tyrosine; however, we did not wish to obtain too many peptides in case small differences in peptides were obscured by the overlapping of too many peptides, e.g., the E<sub>β</sub><sup>s</sup> polypeptide has 7 tyrosine-, 15 leucine-, and 9 phenylalanine-labeled tryptic peptides.

In this peptide map study, we radiolabeled with a single amino acid (usually phenylalanine) because we wished to compare the tryptic maps of I<sub>a</sub> polypeptides. Using this method, small differences in two very similar polypeptides can be demonstrated if radiolabeled peptides are involved. On the other hand, small dissimilarities may go undetected if the structural diversity resides only in unlabeled peptides. Nevertheless, this approach, using a single amino acid as label, is less complex than incorporating several radiolabeled amino acids simultaneously into I<sub>a</sub> proteins. We have observed that the efficiency of biosynthetic incorporation of radiolabels into I<sub>a</sub> polypeptides varies both with the individual mouse used and the particular batch of amino acid; moreover, the relative specific activities of amino acids vary between batches. Therefore, to avoid the necessity of an independent control to measure the relative incorporation of different amino acids into different lymphocytes, we chose to label each preparation with only one amino acid.

Fig. 5 shows the phenylalanine-labeled tryptic peptide maps obtained for A<sub>α</sub> and A<sub>β</sub> polypeptides. Figs. 6 and 7 show the peptide maps used to map the E<sub>β</sub> polypeptides.

*HPLC Mapping of Polypeptides Isolated by 1D-SDS-PAGE and IEF.* To determine whether the invariant protein is structurally related to the I<sub>a</sub> polypeptides, we compared the [<sup>35</sup>S]methionine-labeled and the [<sup>3</sup>H]phenylalanine-labeled peptide maps of A<sub>α</sub><sup>s</sup>, A<sub>β</sub><sup>s</sup>, E<sub>α</sub><sup>k</sup>, and E<sub>β</sub><sup>s</sup> with those of I<sub>i</sub>.

The procedure for isolating the I<sub>i</sub> protein is more complex than for isolating the A<sub>α</sub>, A<sub>β</sub>, E<sub>α</sub>, and E<sub>β</sub> polypeptides, which can be obtained directly from 1D-SDS-PAGE gels. The slices of SDS gels containing the invariant polypeptide are contaminated with an acidic polypeptide (probably incompletely processed forms of A<sub>α</sub> and E<sub>α</sub>; see Fig. 3, panel iv). Fig. 8, panel ii illustrates the results of peptide mapping the proteins of ~31,000 mol wt (intermediate between A<sub>α</sub> and A<sub>β</sub>) containing the invariant polypeptide and a form of the A<sub>α</sub> polypeptide. When compared with panel i, the peptide map of pure A<sub>α</sub>, peptides from both A<sub>α</sub> and the invariant polypeptide are clearly visible. Therefore, we obtained pure invariant polypeptide from A<sup>s</sup> and E<sup>k</sup> immunoprecipitates first by separating the protein of the correct molecular weight on

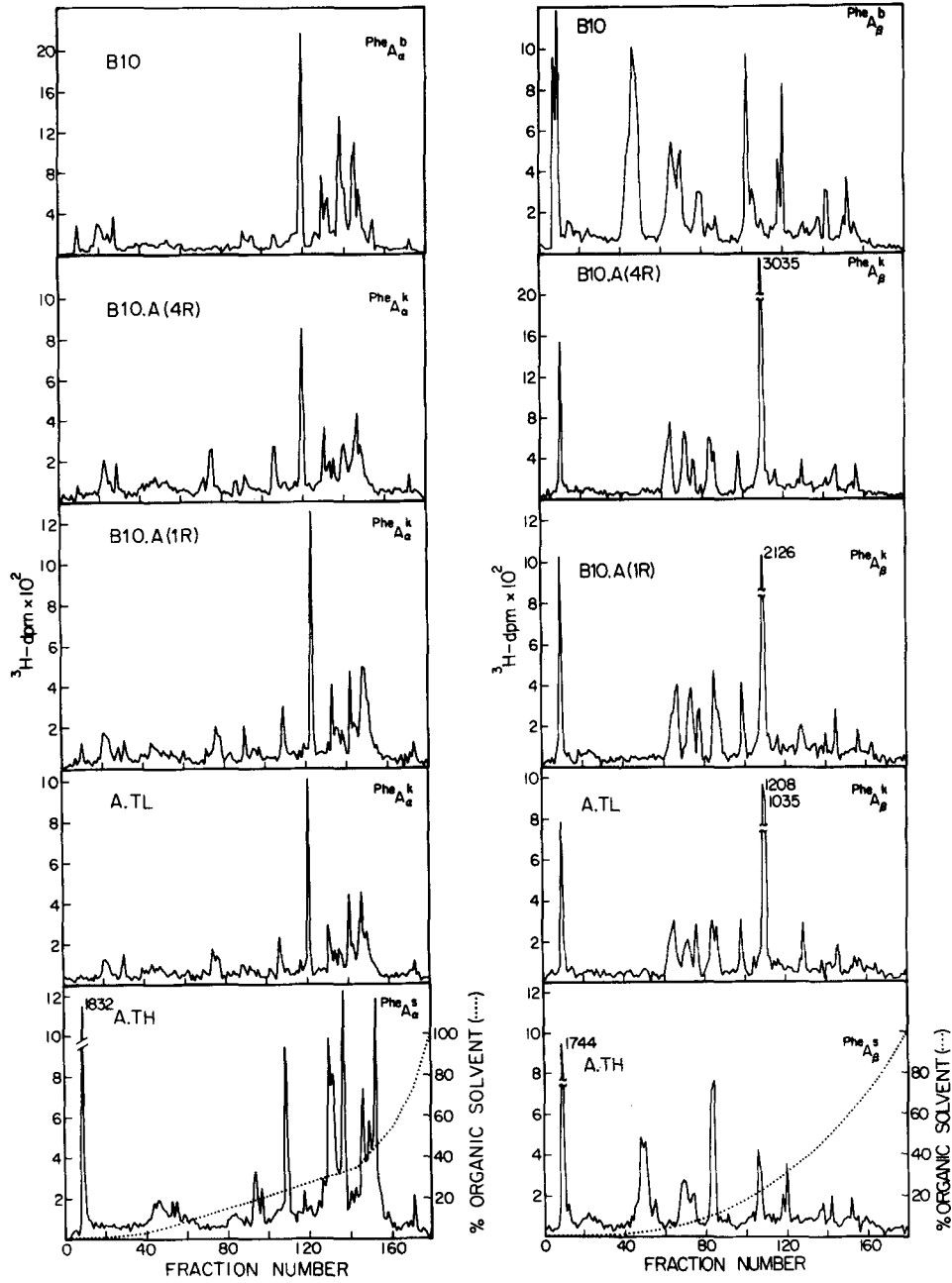


FIG. 5. HPLC tryptic peptide maps of [<sup>3</sup>H]phenylalanine-labeled A<sub>α</sub> (left panels) and A<sub>β</sub> (right panels) Ia polypeptides. The mouse strains from which the maps originate are indicated in the top left corners. Radioactivity is plotted on the ordinates, fraction number of the abscissae. (· · ·) denotes the concentration gradient of organic solvent used for the maps.



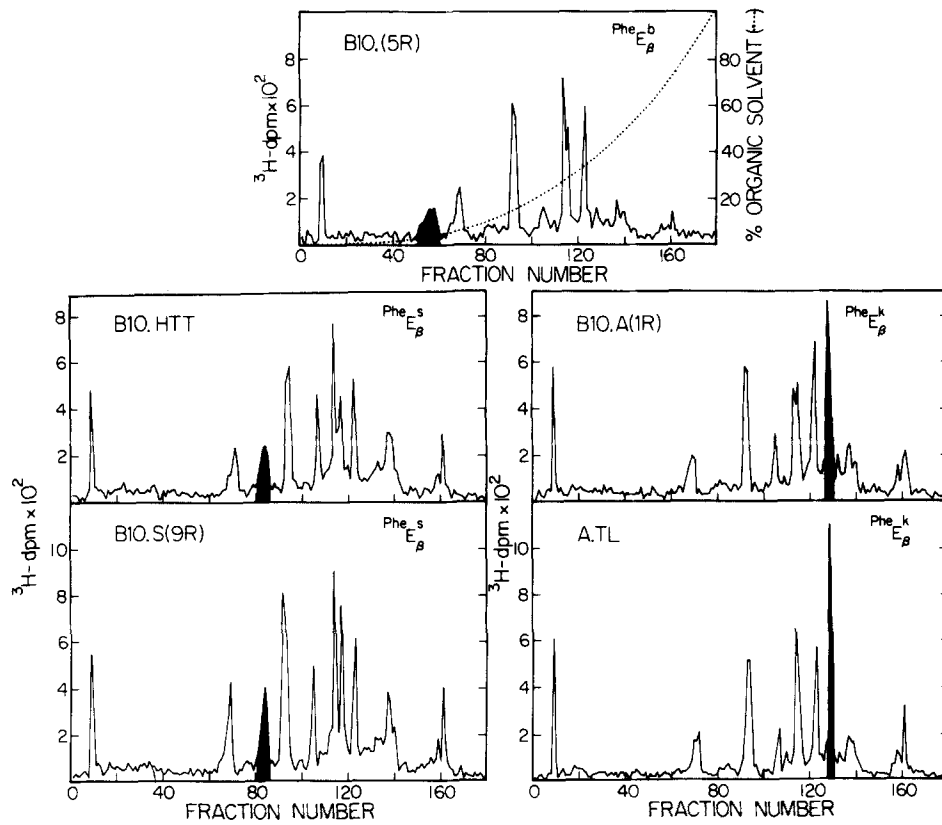


FIG. 6. HPLC tryptic peptide maps of [<sup>3</sup>H]phenylalanine-labeled E<sub>β</sub> polypeptides. Peaks shaded in black represent peptides which are unique to one particular haplotype. See legend to Fig. 5.

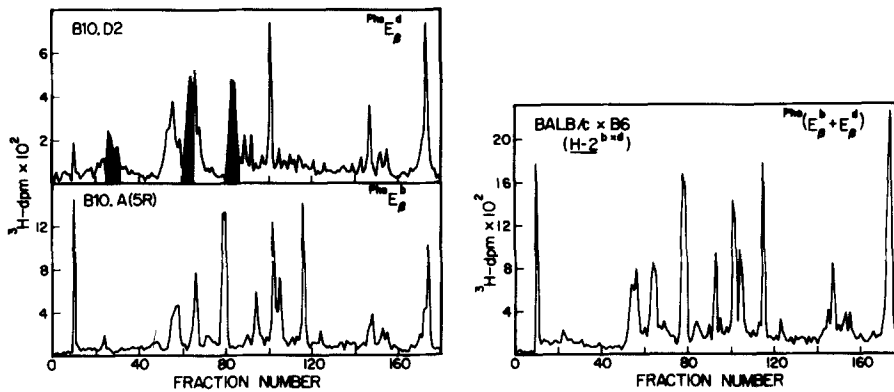


FIG. 7. HPLC tryptic peptide maps of [<sup>3</sup>H]phenylalanine-labeled E<sub>β</sub> polypeptides. Peaks shaded in black represent peptides which are unique to one particular haplotype. The organic solvent gradient is the same as that used for the A<sub>n</sub> peptide maps. See legend to Fig. 5.

1D-SDS-PAGE gels and then by isolating the basic polypeptide from an IEF gel. The [<sup>35</sup>S]methionine-labeled tryptic peptide maps of these two proteins are shown in Fig. 9 and appear to be identical. In these peptide maps of I<sub>i</sub>, eight methionine-labeled

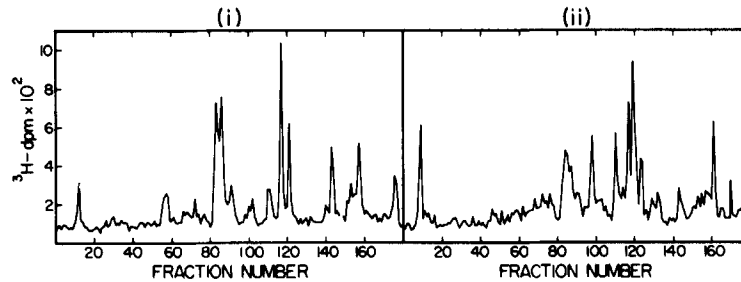


FIG. 8. HPLC tryptic peptide maps of [ $^3\text{H}$ ]leucine-labeled polypeptides from A.TL strain separated on a Zorbax-ODS column. Panel i, pure  $A_a^k$  (~34,000 mol wt); panel ii, invariant polypeptide together with an 'incomplete' form of  $A_a^k$  (~31,000 mol wt). The organic solvent gradient is the same as that used for the  $A_a$  peptides. See legend to Fig. 5.

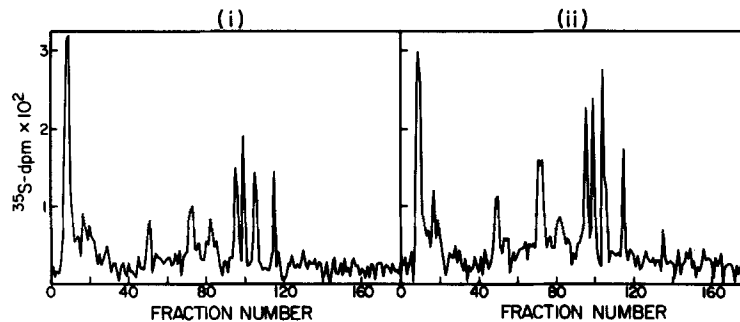


FIG. 9. HPLC tryptic peptide maps of [ $^{35}\text{S}$ ]methionine-labeled invariant polypeptides isolated from a B10.HTT cell lysate by immunoprecipitation, using anti- $A^a$  (left) and anti- $E^k$  sera (right). The polypeptides were separated using SDS-PAGE and IEF. Radioactivity is plotted on each ordinate, the fraction number is plotted on the abscissae. The organic solvent gradient is the same as that used for the  $A_a$  peptide maps.

peptides are separated by the column, whereas in the maps of  $A_a$ ,  $A_\beta$ ,  $E_a$ , and  $E_\beta$ , only 2–3 methionine-labeled peptides are obtained. In contrast, in the [ $^3\text{H}$ ]phenylalanine-labeled tryptic peptide maps, the invariant protein possesses only two labeled peptides, whereas the  $I_a$  polypeptides yield 9–11 labeled peptides. Based on this evidence, we conclude that  $I_i$  is not a precursor of the  $A_a$ ,  $A_\beta$ ,  $E_a$ , and  $E_\beta$  polypeptides, and that none of the  $A_a$ ,  $A_\beta$ ,  $E_a$ , or  $E_\beta$  polypeptides is a precursor of  $I_i$ .

### Discussion

*The Invariant Polypeptide, a Methionine-rich Protein That Specifically Co-immunoprecipitates with  $I_a$  Molecules, in an Association Whose Strength Varies with Haplotype, Is Not a Precursor of Either the  $A_a$ ,  $A_\beta$ ,  $E_a$ , or  $E_\beta$  Polypeptides.* Our electrophoretic analyses of  $I_a$  immunoprecipitates using [ $^3\text{H}$ ]phenylalanine and [ $^{35}\text{S}$ ]methionine show that a third polypeptide,  $I_i$ , described originally by Jones et al. (21), is always isolated under the normal immunoprecipitating conditions described by Cullen and Schwartz (25). Under partially denaturing conditions (0.5% SDS), however, the association of  $I_i$  with  $A^a$  molecules is much stronger than with  $A^k$  molecules.

The amino acid composition of the invariant polypeptide is unusual. It has at least eight methionine-labeled tryptic peptides, in contrast to the  $I_a$  polypeptides, which have 2–3 methionine-labeled peptides (a more common number for proteins in the

30,000-mol wt range). The I<sub>i</sub> polypeptide has only two phenylalanine-labeled tryptic peptides, compared to the 9–11 labeled peptides obtained from the I<sub>a</sub> polypeptides, and as judged from its pI (~8), it also has numerous lysine and/or arginine residues. It is not surprising, therefore, that our peptide map studies demonstrate that the invariant polypeptide is not a precursor of any I<sub>a</sub> polypeptide, A<sub>α</sub>, A<sub>β</sub>, E<sub>α</sub>, or E<sub>β</sub>.

The significance of the interaction of the invariant polypeptide with I<sub>a</sub> molecules is not understood at present, but it may play an as yet undefined physiological role in the functioning of I<sub>a</sub> molecules in the immune response. To date, experimental evidence suggests that I<sub>i</sub> is not polymorphic and, therefore, no conclusions can be drawn about the chromosomal location of the gene encoding the invariant polypeptide.

*The Genes Encoding the I<sub>a</sub> Polypeptides Have Been Mapped Using HPLC Comparative Tryptic Peptide Analyses*

THE GENES ENCODING THE A<sub>α</sub>, A<sub>β</sub>, AND E<sub>β</sub> POLYPEPTIDES MAP TO THE I-A SUBREGION. In Fig. 5, we have compared the phenylalanine-labeled peptide maps of A.TL, B10.A(4R), and B10.A(1R), and have shown that the A<sub>α</sub><sup>k</sup> and A<sub>β</sub><sup>k</sup> polypeptides appear identical to one another but differ from the A<sub>α</sub> and A<sub>β</sub> polypeptides of the H-2<sup>b</sup> and H-2<sup>g</sup> haplotypes of B10 and A.TH. The genes encoding the A<sub>α</sub> and A<sub>β</sub> polypeptides must therefore lie to the left of the I-B subregion and to the right of the H-2K region, i.e., in the I-A subregion. A new subregion, I-N, located between H-2K and I-A, has recently been defined by the proliferative response of the A.TL strain in mixed lymphocyte reactions (39). Our peptide map data on the A.TL I<sub>a</sub> molecules show that both the A<sub>α</sub> and A<sub>β</sub> polypeptides appear identical to those of the H-2<sup>k</sup> haplotype and, therefore, the genes encoding them must map to the I-A and not the I-N subregion.

Several laboratories have reported that the gene encoding the E<sub>β</sub> polypeptide maps the I-A subregion, which agrees with our data (34–36, 38, 40). In Fig. 6, the peptide map of E<sub>β</sub> from B10.A(5R) differs from those of B10.A(1R) and A.TL (which are identical to each other), and from those of B10.HTT and B10.S(9R) (which also are indistinguishable from one another). Thus, E<sub>β</sub> varies depending on the haplotype of the I-A and not the I-E subregion. To formally prove the gene encoding E<sub>β</sub> lies in the I-A subregion, we have also examined the structure of the E<sub>β</sub> polypeptide in strains having the same I-A subregion (I-A<sup>b</sup>) but differing in the origin of their I-E subregions (I-E<sup>k</sup> and I-E<sup>d</sup>). Because of the lack of a recombinant mouse having an I-A<sup>b</sup>E<sup>d</sup> haplotype, we have used an F<sub>1</sub> hybrid mouse having the H-2<sup>bxd</sup> haplotype. This F<sub>1</sub> mouse would be expected to express on its cell surface the hybrid I<sub>a</sub> molecules, E<sub>α</sub><sup>d</sup>E<sub>β</sub><sup>d</sup> + E<sub>α</sub><sup>d</sup>E<sub>β</sub><sup>b</sup> (38, 40, 41). Our experiment is designed to answer the following question: is this E<sub>β</sub><sup>b</sup> polypeptide in the E<sub>α</sub><sup>d</sup>E<sub>β</sub><sup>b</sup> molecule the same as or different from the E<sub>β</sub><sup>b</sup> polypeptide in a B10.A(5R) mouse (E<sub>α</sub><sup>k</sup>E<sub>β</sub><sup>b</sup>)? Fig. 7 shows that these two polypeptides are, in fact, identical, and formally proves that the E<sub>β</sub> polypeptide is encoded by a gene mapping to the I-A subregion.

THE GENE ENCODING THE E<sub>α</sub> POLYPEPTIDE MAPS TO THE I-E SUBREGION. Because the I<sub>a</sub> molecules, E<sub>α</sub>E<sub>β</sub>, are immunoprecipitated using anti-E subregion reagents, the E<sub>α</sub> polypeptide must map to the I-E subregion, as the E<sub>β</sub> polypeptide maps to the I-A subregion. Although 2D electrophoresis studies by Jones (31) indicate that E<sub>α</sub><sup>d</sup> differs from E<sub>α</sub><sup>k</sup>, we found no detectable differences between the [<sup>3</sup>H]phenylalanine-labeled

peptide maps of  $E_{\alpha}^d$  and  $E_{\alpha}^k$  from B10.D2 and B10.A(5R) strains. This agrees with the data of Silver et al. (42), who analyzed the peptides using IEF. Cook et al. (14) report 93% identity of tryptic peptides when multiple amino acids are used as radiolabels.

*The  $E_{\beta}$  Polypeptides of the  $H-2^{k,b,s}$  Haplotypes Are More Chemically Similar to Each Other than to the  $E_{\beta}$  Polypeptides of the  $H-2^d$  Haplotype.* Fig. 6 illustrates that the  $E_{\beta}$  polypeptide from B10.A(5R), B10.A(1R), A.TL, B10.HTT, and B10.S(9R) have all but one of their phenylalanine-containing peptides in common (8 out of 9), and that each haplotype appears to have one unique peptide per haplotype (shaded black). Because peptide mapping tends to exaggerate differences between homologous proteins, this means that the  $E_{\beta}^{k,b,s}$  polypeptides are very similar; in fact, the  $E_{\beta}^k$  and  $E_{\beta}^s$  chains are indistinguishable by 2D electrophoresis, although they are chemically distinct by peptide mapping. In contrast, the  $E_{\beta}^d$  chain shares only 6 out of 9 of its phenylalanine-labeled peptides with the  $E_{\beta}^{k,b,s}$  polypeptides (Fig. 7), which agrees with our previous studies using tyrosine- and leucine-labeled proteins (18).

This observation correlates beautifully with the observations of Lerner et al. (43), who have made a monoclonal antibody that reacts with Ia molecules composed of  $E_{\alpha}$  and  $E_{\beta}^{k,b,s}$ , but not  $E_{\alpha}$  and  $E_{\beta}^d$ . It also is consistent with the serological work on Ia hybrid molecules of Lafuse et al. (44), who have shown that  $E_{\beta}^b$  and  $E_{\beta}^s$  share, together with  $E_{\beta}^k$ , the determinant defining the specificity Ia.22. The  $E_{\beta}^{k,b,s}$  polypeptides do not express specificity Ia.23, which resides on the  $E_{\beta}^d$  chain.

*$E_{\beta}$  Polypeptides in Ia Hybrid Molecules in  $F_1$  Mice Turn Over at Different Rates.* Fig. 7 illustrates that in the peptide map of the  $H-2^{bx^d}$  mouse the ratio of peptides unique to  $E_{\beta}^d$  compared to those unique to  $E_{\beta}^b$  is not the 1:1 ratio that would be expected assuming simple gene-dosage effects. The  $E_{\beta}^b$  polypeptide is more biosynthetically labeled than the  $E_{\beta}^d$  chain, and we have confirmed this observation independently by 2D electrophoresis, which showed that the  $E_{\beta}^b$  spot was much more intense than the  $E_{\beta}^d$  spot. This differential labeling is not due to differences in the phenylalanine content of these proteins, because  $E_{\beta}^b$  and  $E_{\beta}^d$  are highly homologous polypeptides, sharing 66% of their phenylalanine-labeled tryptic peptides and having at least 7 out of 8 identical residues in their partial N-terminal amino acid sequences (17).

Recently, Emerson et al. (45) have studied the biosynthesis and turnover of the  $E_{\beta}$  polypeptides in homozygous ( $H-2^k$ ) mice. They find that whereas the  $E_{\beta}$  chains are synthesized more rapidly than  $E_{\alpha}$  chains, the  $E_{\alpha}E_{\beta}$  molecules, once assembled, turn over at the same rate.

We should stress that experiments involving biosynthetic labeling cannot address questions involving the total amount of Ia protein expressed by a cell. However, these intriguing observations on the differential labeling of  $E_{\beta}$  polypeptides in  $F_1$  mice, together with the observations of Emerson et al., underline the fact that the control of synthesis of Ia molecules may itself be complex and could possibly be haplotype-dependent.

Thus, the role that Ia molecules play in immune responsiveness may depend not only on structural polymorphism and the diversity that can be generated by hybrid molecules, but also on the rate at which these molecules can be synthesized and turned over. New insights into this problem will undoubtedly be obtained when the Ia genes themselves can be examined and their regulatory mechanisms are investigated.

### Summary

We demonstrate that an invariant polypeptide, first described by Jones et al. (21), co-immunoprecipitates with our Ia molecules, that its interaction with Ia polypeptides varies with haplotype, and that it is not a precursor of the A $_{\alpha}$ , A $_{\beta}$ , E $_{\alpha}$ , or E $_{\beta}$  polypeptides. We also show that the polypeptides that we have previously characterized are contaminated with very little, if any, invariant protein. Further, we have used our high-pressure liquid chromatography tryptic peptide map technique to formally map the genes encoding A $_{\alpha}$ , A $_{\beta}$ , and E $_{\beta}$  to the *I-A* subregion using recombinant and F $_1$  hybrid mice.

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