EVIDENCE FOR TWO HOMOLOGOUS, BUT NONIDENTICAL, Ia MOLECULES DETERMINED BY THE I-EC SUBREGION*

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The I region of the mouse H-2 complex is divided into subregions, I-A, I-B, I-F, I-E, and I-C, that are marked by the Ia-1, Ia-2, Ia-4, Ia-5, and Ia-3 loci, respectively (1, 2). The Ia-1, Ia-5, and Ia-3 locus structural products are Ia antigens, a group of polymorphic, cell-surface glycoproteins that consist of two noncovalently associated polypeptide chains of mol wt \( \approx 30,000-35,000 \) (\( \alpha \)-subunit) and 25,000–30,000 (\( \beta \)-subunit) (3, 4).

Sequential precipitation analyses have previously shown that an Ia-5 locus product is independently precipitable from a second, distinct Ia molecule determined by either the Ia-5 or Ia-3 locus (5). More recently, it was reported (6) that Ia.22 and Ia.23, private specificities of the I-E subregion of the H-2^k and H-2^d haplotypes, respectively, are coprecipitable with Ia.7, formerly considered to be a public specificity of the I-C subregion. To explain this finding, it was proposed that Ia.7 be assigned to the I-E subregion; and that Ia.6, which is difficult to detect serologically, is the only specificity determined by the I-C subregion. It is implied in the latter studies that only one Ia molecule controlled by a locus mapping between the I-J and S regions is detectable by immunoprecipitation.

To resolve this apparent discrepancy, we have performed sequential precipitation, two-dimensional (2D)\(^1\) gel electrophoresis, and peptide mapping analyses of I-EC\(^2\) subregion products. We demonstrate here the presence of two Ia molecules in the I-EC subregion that are identical in molecular size and charge, but differ by \( \approx 20\% \) in their peptides obtained by partial digestion with Staphylococcus aureus protease V8.

Materials and Methods

Immunoprecipitation. The immunoprecipitation of B10.A(3R) \(^{35}\)S-methionine-labeled, Nonidet-P-40- (NP-40, Particle Data, Inc., Elmhurst, Ill.) solubilized, lentil-lectin column-bound and eluted spleen cell lysates was carried out using the Cowan I strain of Staphylococcus aureus (S. aureus C) as previously described (7, 8). The production and serological characterization of the B10.S(7R) anti-B10.HTT (anti-I-E\(^k\), I-C\(^k\), S\(^k\), G\(^k\)) and (B10 \( \times \) HTT)F\(_1\) anti-B10.A(5R) (anti-I-J\(^k\), I-E\(^k\), I-C\(^d\), S\(^d\), G\(^d\)) antisera used were previously reported (5).

* Supported by the Medical Research Council of Canada grants MA 5729 and MA 6004.
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\(^1\) Abbreviations used in this paper: 1D, one-dimensional; 2D, two-dimensional; NP-40, Nonidet P-40; S. aureus C, Cowan I strain of Staphylococcus aureus; SDS, sodium dodecyl sulfate.
\(^2\) Due to the difficulties encountered by several investigators in the serological and immunochemical resolution of I-E and I-C subregion products, Ia molecules determined by this chromosome segment are presently collectively termed I-EC subregion products.
Gel Electrophoresis. Immunoprecipitated samples were eluted from *S. aureus* C by boiling for 1 min in the presence of 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol and then electrophoresed under reducing conditions on a one-dimensional (1D), 10% SDS-polyacrylamide slab gel (13 × 18 × 0.1 cm) according to Laemmli (9), as previously described (8). Alternatively, samples were eluted from *S. aureus* C during 1 min at room temperature in sample lysis buffer (9.5 M urea, 2% [wt/vol] NP-40, 2% Ampholine [1.6%, pH 5-7; and 0.4%, pH 3-10; LKB Produkter, Bromma, Sweden], and 5% 2-mercaptoethanol) and were then analyzed by 2D gel electrophoresis following the O'Farrell method (10), as previously reported (8). The radioactivity in the 2D gels was revealed by fluorography after the impregnation of the gels with PPO in dimethyl sulfoxide (11).

Peptide Mapping. Peptide mapping by limited proteolysis in SDS gels was carried out according to Cleveland et al. (12), as previously described (8, 13). Briefly, 35S-methionine-labeled, immunoprecipitated samples were eluted from *S. aureus* C and divided into aliquots containing four-fifths and one-fifth of the sample radioactivity. These aliquots were electrophoresed under reducing conditions in adjacent, parallel tracks of a 1D, 10% SDS-polyacrylamide slab gel, as outlined above. The positions of the EC₃⁻ and EC₇⁻ labeled polypeptide chains were determined in the track containing one-fifth of the sample by the liquid scintillation counting of 2-mm gel slices solubilized in Protosol-Omnifluor-toluene (New England Nuclear, Boston, Mass.) (8). Gel slices in the track containing four-fifths of the sample and corresponding to the leading portion of the EC₃ peak and the trailing portion of the EC₇ peak were cut; equilibrated; transferred to sample wells of a second, 15% SDS-polyacrylamide slab gel; and digested with *S. aureus* protease V8 (0.05 µg) during electrophoresis (9, 13). The radioactivity in 2-mm gel slices was quantitated by liquid scintillation spectrometry as mentioned above.

Results

2D-gel-electrophoretic studies were carried out on radiolabeled, immunoprecipitated Ia antigens determined by the I-EC subregion. B10.A(3R) lysates were treated with B10.S(7R) anti-B10.HTT (anti-I-E₆, I-C₆, S₄, G₄) and (B10 × HT1)F₁ anti-B10.A(5R) (anti-I-A₆, I-C₆, S₄, G₄) antisera. Identical 2D gel patterns of the Ia molecules immunoprecipitated with these sera were obtained (Figs. 1 A and D). The fluorograms show the presence of several spots in the 25,000-35,000-mol wt range that correspond to biosynthetic intermediates of Ia antigens that display altered amounts of glycosylation (14). The Ia spot pattern obtained with the (B10 × HT1)F₁ anti-B10.A(5R) serum is similar to that previously observed, and most likely is representative of at least one I-EC subregion product and at least one I-A subregion product (15). It should also be noted that the 2D gel patterns of spots in the Ia region of lentil-lectin bound and eluted lysates immunoprecipitated with the same batches of the B10.S(7R) anti-B10.HTT and (B10 × HT1)F₁ anti-B10.A(5R) sera are identical to the profiles shown in Figs. 1 A and 1 D. Non-Ia spots, apparent in the actin and Ia regions of Fig. 1 D, are absent from the gel patterns of the lentil-lectin, purified lysates.

Each antiserum used here quantitatively removes from the lysate all its Ia reactivity in a single precipitation. No Ia-like spots were observed when samples were first treated with either B10.S(7R) anti-B10.HTT or (B10 × HT1)F₁ anti-B10.A(5R); the supernate of this precipitate was further treated with the same anti-Ia serum (Figs. 1 B and 1 E). However, treatment of the lysate, first with B10.S(7R) anti-B10.HTT and, second with (B10 × HT1)F₁ anti-B10.A(5R) results in the independent precipit-
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Fig. 1. 2D gel electrophoresis of immunoprecipitated, ^35S-methionine-labeled B10.A(3R) spleen cell lysates. Lysates from 10^7 spleen lymphocytes were reacted with either B10.S(7R) anti-B10.HTT (A) or (B10 X HTT)F1 anti-B10.A(5R) (D) and *S. aureus* C. Sequential precipitations were performed using either B10.S(7R) anti-B10.HTT (B) or (B10 X HTT)F1 anti-B10.A(5R) (E) in both the first and second steps. Sequential precipitations were also carried out by treatment of lysates, first with (B10 X HTT)F1 anti-B10.A(5R), and second with B10.S(7R) anti-B10.HTT (C), or in the reverse order (F). Immunoprecipitates were analyzed by isoelectric focusing in the first dimension (left to right) and by SDS-polyacrylamide slab gel electrophoresis in the second dimension (top to bottom). The basic (pH 7.5) end is at the left and the acid (pH 4.5) end is at the right. About 95,500 cpm (A), 4,970 cpm (B), 7,615 cpm (C), 72,460 cpm (D), 5,870 cpm (E), and 27,100 cpm (F) were loaded onto the focusing gels. The slab gels were fluorographed and exposed to SB-54 x-ray film (Eastman Kodak Co., Rochester, N. Y.) for 3 wk. Only the 20,000-50,000-mol wt range of the slab gels is shown. Actin (mol wt = 43,000) is designated by the letter "a." Ia spots are enclosed. The three basic Ia spots at the lower left of the enclosed region may be I-A^k^ products (15; Discussion). The results of three experiments are shown.

Coprecipitation of two distinct Ia molecules (Fig. 1 F). Coprecipitation occurs when the order of serum treatment is reversed (Fig. 1 C). These data are identical to those we previously reported using 1D gel electrophoresis and sera that was raised in the same strain combinations, but collected from a different set of mice (5). Moreover, they suggest that the I-EC subregion codes for two Ia molecules that possess the same molecular size and net charge, and yet are separable by immunoprecipitation. Differences in these I-EC products, therefore, likely reside in their protein moiety and not in their carbohydrate moiety (3).

To determine whether these two Ia molecules do indeed differ, perhaps in their neutral amino acids, we analyzed their primary sequence homology by a limited
proteolysis peptide mapping technique (12). B10.A(3R) immunoprecipitates obtained with either B10.S(7R) anti-B10.HTT or (B10 × HTT)F₁ anti-B10.A(5R) were electrophoresed under reducing conditions in parallel tracks of a 1D, 10% SDS-polyacrylamide slab gel. Representative profiles of the separated EC₅ₐ and EC₆ₖ polypeptide chains detected by the two sera are shown in Fig. 2. It is evident that the α- and β-subunits of I-EC subregion products are not well resolved; they cannot be separated from each other as well as the subunits of an I-A subregion product (3, 16). The EC₅ₐ chains precipitated with both antisera are of the same molecular size; the same is true for the respective EC₆ₖ chains.

The EC₅ₐ and EC₆ₖ polypeptides present in gel slices cut from the different tracks of the slab gel (Fig. 2) were partially digested in situ with *S. aureus* protease V8; the peptide maps obtained are presented in Fig. 3. Figs. 3A and B demonstrate that one of eight EC₅ₐ peptides and two of seven EC₆ₖ peptides, respectively, differ between the Ia molecules detected with the antisera used here. All of the peptides comprising the molecule detected with B10.S(7R) anti-B10.HTT are present in the molecule precipitated with (B10 × HTT)F₁ anti-B10.A(5R). The three differing peptides found are present in the latter molecule and absent from the former one. The (B10 ×

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5 While immunoprecipitated α and β polypeptides are for simplicity referred to here as the EC₅ₐ and EC₆ₖ chains, it should be noted that only one of these chains may be an I-ECₖ product, whereas the other one may be an I-Aₖ product, which yields a different spot pattern from the I-Aₖ product of the H-2* and H-2₃ haplotypes. The group of three most basic Ia spots shown in Figs. 1 A, D, and F may be controlled by the I-Aₖ subregion.

6 Delovitch, T. Unpublished observations.
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Fig. 3. Peptide maps of ECαk and ECβk polypeptides. 35S-methionine-labeled B10.A(3R) spleen cell lysates were immunoprecipitated (A and B) with either (B10 × HTI)F1 anti-B10.A(5R) (---) or B10.S(7R) anti-B10.HTT(--), or sequentially (C and D), first with B10.S(7R) anti-B10.HTT, and second with (B10 × HTI)F1 anti-B10.A(5R). They were then electrophoresed on a 10% SDS-polyacrylamide slab gel as illustrated in Fig. 2. The ECα (A and C) and ECβ (B and D) polypeptides were recovered in gel slices as indicated and then partially digested with 0.5 µg S. aureus protease V8 during electrophoresis on a 15% SDS-polyacrylamide slab gel. The positions of the various Coomassie-Blue-stained marker proteins, phosphorylase a (94,000 mol wt), bovine serum albumin (68,000 mol wt), actin (43,000 mol wt), DNase I (31,000 mol wt), and lysozyme (14,000 mol wt), run on the same slab gel are indicated by arrows. Peaks of radioactivity appearing in fractions 1-20 represent undigested and aggregated protein. The peak appearing in fractions 25-30 of panels B and D presumably represents undigested ECαk chains (28,000 mol wt). The results of two experiments are shown.

Discussion

Evidence is presented here for the presence of two structurally homologous, but nonidentical, I-EC subregion products that may be immunoprecipitated independently. The α- and β- subunits of these two, distinct Ia molecules possess the same molecular size and charge, yet differ with respect to their S. aureus protease V8 peptides. By reference to the current I-subregion distribution of Ia antigenic specificities (6), it is likely that both of the Ia molecules characterized here are determined by the Ia-5 locus in the I-E subregion. The B10.S(7R) anti-B10.HTT serum does not detect an I-C subregion product (5). Thus far, the detection of Ia-3 locus products controlled by the I-C subregion has proven difficult (6, 15, 17).

A comparison of the peptide maps of the two Ia molecules characterized here
indicate that 3 of the 15 peptides present in a molecule precipitable by (B10 × HTI)F1 anti-B10.A(5R) are absent from the molecule detected with B10.S(7R) anti-B10.HTT. These analyses suggest that the two I-EC\(^k\) products possess \(\approx80\%\) shared peptides, and that they differ presumably by multiple amino acid residues. The 2D gel profiles presented herein support the idea that such peptide differences are attributable primarily to neutral amino acid substitutions. It may also be deduced from these peptide maps that EC\(_{\alpha}^k\) and EC\(_{\beta}^k\) polypeptides of a given Ia molecule share, at most, 10–15\% of their \textit{S. aureus} protease V8 peptides. Due to the limited proteolysis technique used here, it is conceivable that the observed peptide differences may underestimate the actual extent of primary sequence variation between these two Ia molecules. Procedures employing complete proteolysis may reveal less structural homology than was observed here. Conversely, the possibility that the number of peptide differences demonstrated here may be reduced or even eliminated upon complete digestion, resulting, thereby, in an increased extent of homology, must also be considered. It therefore will be necessary in the future to utilize alternate proteolysis conditions by employing other enzymes and lengthier times of digestion.

Despite the possible limitations of the peptide mapping technique used, the data presented in this study agree closely with those obtained by partial NH\(_2\)-terminal amino acid sequencing of I-EC subregion products (18–21). It has been reported that EC\(_{\alpha}^d\) and EC\(_{\beta}^d\) polypeptides possess a single major sequence but also exhibit minor sequence heterogeneity (21). I-EC subregion products may therefore bear homologous, but distinct, sequences. The \(\alpha\)- and \(\beta\)-polypeptides of either an EC\(_k\) or an EC\(_k^d\) molecule show no identity in the residues identified and compared thus far. EC\(_{\alpha}^d\) and EC\(_{\alpha}^k\) polypeptides are identical in eight positions that can be compared, whereas EC\(_{\beta}^d\) and EC\(_{\beta}^k\) chains differ in two of the seven (29\% difference) positions compared. Hence, both preliminary peptide mapping and NH\(_2\)-terminal amino acid sequencing suggest the presence of multiple (\(\geq2\)) Ia molecules in the I-EC subregion. These structural characterizations also demonstrate that such molecules likely express more \(\beta\)-chain that \(\alpha\)-chain sequence variations.

Thus, the above sets of observations imply that \(\alpha\)- and \(\beta\)-genes mapped to the I-EC subregion of a particular H-2 haplotype may have evolved from a common ancestral gene that had undergone minimal somatic mutation during gene duplication throughout evolution. Such an explanation of the data is compatible with the gene duplication model of the H-2 complex (22). Whether this genetic event actually occurred, and whether this postulated pattern of divergence is applicable to \(\alpha\) and \(\beta\) genes of other I subregions, can only be determined from further structural analyses of many Ia molecules.

An additional level of genetic and structural complexity of I-region products to that presented above must be considered. Jones et al. (15) recently found that anti-I-E\(^k\) antibodies coprecipitate two Ia polypeptide chains; one chain is coded for by a locus in I-E, and the other chain (A\(_\alpha\)) is determined by a locus in I-A. They postulated that complementation between these two loci is necessary for the detection at the cell surface of the A\(_\alpha\) polypeptide in a molecular complex with the I-E product, and that the I-E locus may regulate the membrane expression of A\(_\alpha\). An extrapolation of these findings to those presented in this study would suggest that the (B10 × HTI)F1 anti-B10.A(5R) serum we used coprecipitates two I-A products and two I-E products. It is, therefore, possible that the EC\(_{\alpha}\) chains of the second molecule shown in Figs. 3 C and
D actually represent an I-E and I-A product, respectively. If this is the case, then both the I-A and I-E subregions of a given haplotype may contain several (at least two) loci that each code for a separate Ia polypeptide chain. As mentioned above, this might imply that Ia chains determined by a particular I-subregion are evolutionarily related, and that this relationship might allow for a high degree of sequence homology to exist among such a subset of proteins. Further, it is conceivable that closely linked genes of the I-A and I-E subregions have evolved in parallel; this might afford a means of selection, from the various possible molecular associations, of those that can give rise to a functionally active Ia molecule. A given I-A product may be able to combine with more than one I-E product of either the same haplotype or a different haplotype. The converse may also occur. Thus, multiple combinatorial joinings of Ia polypeptide chains of various subregions and haplotypes could potentially generate a wide diversity of antigenically distinct Ia molecules.

It should be mentioned that coprecipitation of different Ia determinants controlled by a particular I-subregion may be obtained if they are present on either the same Ia molecule or on separate, but structurally homologous, molecules, as previously suggested (5). Ia determinants controlled by the I-A subregion of a given haplotype have, thus far, all been coprecipitable (3, 5). However, it is still possible that the I-A subregion codes for more than one Ia molecule. The type of I-A products being considered here would consist of Al and A2 chains, and these molecules may exhibit structural relationships similar to the two I-EC subregion products characterized.

Finally, it is interesting to note that a parallel may be drawn here between the results obtained from the multigenic system of Ia antigens and those genes coding for different serum albumins. Bovine serum albumin and human serum albumin display 80-90% sequence homology (23), yet give rise to different Staphylococcus aureus protease V8 peptide patterns. The type of structural variations observed here may therefore be applicable to the products of several different families of genes.

Summary

Sequential immunoprecipitation, two-dimensional gel electrophoresis and peptide mapping analyses of B10.A(3R), 35S-methionine-labeled, I-EC subregion products were performed. Evidence is presented here for the presence of two structurally homologous, but nonidentical, gene products of the I-EC subregion. These two Ia molecules are independently immunoprecipitable, identical in molecular size and charge, but differ by ±20% in their peptides obtained by partial digestion with Staphylococcus aureus protease V8.

We thank Judy Biggin and Catherine Ingram for their expert technical assistance, and Jane Jeffery for her cheerful secretarial assistance.

Received for publication 13 September 1978.

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