

# Mouse invariant chain $\gamma$ exhibits structural homology to both polymorphic subunits of the $\alpha,\beta$ -core complex of I-A<sup>k</sup> antigens

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The 3 major constituents of the I-A<sup>k</sup> subregion-associated complex  $\alpha$ ,  $\beta$  and  $\gamma$  were obtained from splenocytes in homogeneous form by differential isolation methods.  $\alpha$ ,  $\beta$  and  $\gamma$  were compared on the primary structural level by enzymatic fragmentation procedures and tryptic peptide map analysis of radiolabeled proteins. The data indicate that the invariant chain  $\gamma$  exhibits extensive structural homology to the polymorphic  $\beta$ -light and the  $\alpha$ -heavy chain. Thus, although not being encoded within the MHC  $\gamma$  appears to belong structurally to the MHC-encoded class II proteins.

MHC class II antigen	Spleen lymphocyte	Radiolabeling	2D O'Farrell gel
	Electroblotting	Comparative peptide analysis	

## 1. INTRODUCTION

Ia antigens are polymorphic integral membrane glycoproteins that are intimately involved in processes of the immune response. Two regions I-A and I-E, located within the MHC of the mouse contain the genes for molecules organized into two polypeptide chain complexes. The I-A-dependent complex consists of the subunits A $\alpha$  (35 kDa) and A $\beta$  (28 kDa) whereas the I-E complex is composed of E $\alpha$  (36 kDa), mapping to the I-E region and E $\beta$  (31 kDa) mapping to the I-A region (review [1]). Intracellularly both  $\alpha$ ,  $\beta$  core complexes are found associated non-covalently with the invariant chain Ii ( $\gamma$ ) (32 kDa), which is a membrane glycoprotein as are the heavy and light chains [2]. The  $\gamma$  chain is not encoded within the MHC [3,4].

Recently we reported that the light chain  $\beta$  of the I-A<sup>k</sup> complex can be separated on one-dimensional (1D) SDS-polyacrylamide gels in the absence of reducing agents into two distinct molecular

species:  $\beta_1$  (27 kDa) and  $\beta_2$  (25 kDa) [5]. The structural comparisons presented here support our notion that  $\beta_1$  and  $\beta_2$  are two conformational variants of the light chain  $\beta$ . Surprising structural similarities are apparent between the invariant chain  $\gamma$  and the polymorphic  $\beta$  chain and to a lesser extent between  $\gamma$  and the  $\alpha$  chain, suggesting that the  $\gamma$  chain is structurally related to the two MHC encoded class II proteins  $\alpha$  and  $\beta$ .

## 2. MATERIALS AND METHODS

Spleen cells from C3H/HeSn mice, raised in our own mouse colony and used at 10–12 weeks of age, were biosynthetically labeled for 4 h with 100  $\mu$ Ci  $\cdot 10^7$  cells<sup>-1</sup> ml<sup>-1</sup> [<sup>35</sup>S] methionine and extracted with NP 40 (Roth, Karlsruhe) as in [6]. Radiolabeled immunoglobulin was removed from the extracts by preabsorption with rabbit anti-mouse Ig antiserum and Pansorbin (Calbiochem, Giessen). The isolation of single polypeptide chains by immunoprecipitation with monoclonal antibody 10–2.16 (anti I-A<sup>k</sup>) [7] and adsorption to protein A-Sepharose (Pharmacia) followed by preparative 1D SDS-PAGE on long (12  $\times$  23 cm) polyacrylamide gradient gels was as outlined

**Abbreviations:** MHC, major histocompatibility complex; TPCK, *N*-tosylphenylalanine chloromethyl ketone; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

before [6]. Fragmentation analysis was done as in [8]. Papain (Boehringer, Mannheim) and *Staph. aureus* V8 protease (Miles) were used for generation of polypeptide fragments. Labeling of free SH-groups of  $\alpha$ ,  $\gamma$ ,  $\beta_1$  and  $\beta_2$  was done by reacting portions  $\sim 10^4$  cpm  $^{35}\text{S}$  of the subunits with iodo- $^{14}\text{C}$ acetic acid (12  $\mu\text{Ci}$ ) (Amersham, Braunschweig) in 100  $\mu\text{l}$  0.01 M Tris-HCl (pH 8) for 1 h. Excess label was removed by microdialysis. The subunits were then completely reduced and alkylated with iodoacetamide according to standard procedures. Tryptic digests were performed by incubating  $2 \times 10^5$  cpm of the  $^{14}\text{C}$ -labeled protein in 100  $\mu\text{l}$  0.1 M  $\text{NH}_4\text{HCO}_3$  (pH 8.0) with 2  $\mu\text{l}$  of 1% TPCK-trypsin in  $\text{H}_2\text{O}$  for 1 h at  $37^\circ\text{C}$  followed by the same amount of enzyme for another 3 h. Peptide mixtures were desalted by lyophilisation and subjected to 2D thin-layer peptide analysis on cellulose plates (20  $\times$  20 cm) (Polygram Cel 300, Machery-Nagel, Düren) applying ascending chromatography in  $\text{H}_2\text{O}/n$ -butanol/pyridine/acetic acid 24:30:20:6 (by vol.) for the first dimension and electrophoresis in  $\text{H}_2\text{O}/\text{pyridine}/\text{acetic acid}$  756:4:40 (by vol.) (600 V at  $10^\circ\text{C}/90$  min) for the second dimension. The dried plates were placed on Kodak XR-5 film for autoradiography. Alternatively, highly purified [ $^{35}\text{S}$ ]methionine-labeled subunits  $\alpha$ ,  $\gamma$  and  $\beta$  were prepared by immunoaffinity chromatography of [ $^{35}\text{S}$ ]methionine labeled C3H spleen cell NP40 extract on Sepharose CL-4B to which monoclonal antibody 10-2.16 was linked [6] followed by 2D O'Farrell electrophoresis [9] and electroblotting onto nitrocellulose [10]. Nitrocellulose bound proteins were reduced and alkylated with iodoacetamide digested with TPCK-trypsin and subjected to 2D thin-layer peptide analysis as above.

### 3. RESULTS AND DISCUSSION

I-A<sup>k</sup> molecules were isolated from [ $^{35}\text{S}$ ]methionine labeled spleen lymphocytes by immunoprecipitation with monoclonal antibody 10-2.16. Specific immunocomplexes separate on long SDS-polyacrylamide gradient gels (fig. 1) in the absence of reducing agents into 4 bands corresponding to heavy chain  $\alpha$ , invariant chain  $\gamma$  and two conformational variants of the light chain  $\beta$ , designated  $\beta_1$ , and  $\beta_2$  [5]. The high resolution of this preparative run permitted the isolation of

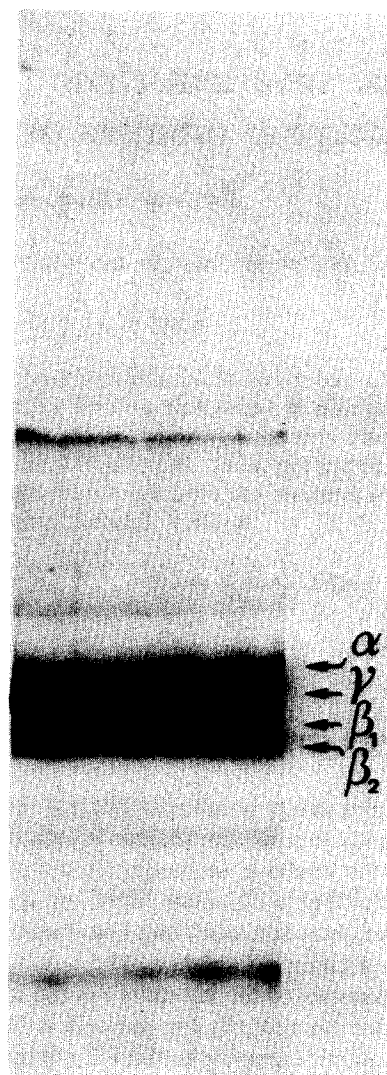


Fig. 1. Preparative SDS-PAGE separation of [ $^{35}\text{S}$ ]methionine labeled I-A<sup>k</sup> antigens in the absence of reducing agents on a linear 5–20% polyacrylamide gradient gel.

homogeneous polypeptides from the wet gel using the autoradiogram as template. Electrophoresis of test aliquots of isolated  $\alpha$ ,  $\gamma$ ,  $\beta_1$  and  $\beta_2$  polypeptides on 2D O'Farrell gels indicated that homogeneity of all four I-A<sup>k</sup> subunits had been achieved (not shown). To elucidate the structural relationship of  $\alpha$ ,  $\gamma$ ,  $\beta_1$  and  $\beta_2$  we subjected the individual polypeptides to a fragmentation analysis [8]. Limited cleavage with papain and *Staph. aureus*

V8 protease, respectively generated large proteolytic fragments which were easily separated on SDS-polyacrylamide slab gradient gels (10–25%) (fig. 2). Similar conclusions can be drawn from both fragmentation patterns. Comparison of the papain and the *Staph. aureus* V8 protease fragments of  $\beta_1$  and  $\beta_2$  shows that both polypeptides share most of the fragments generated, supporting the notion that both chains possess a very similar if not identical primary structure. Furthermore, the  $\alpha$  polypeptide shows striking homology with the  $\beta$  chains and, to a lesser extent, with the  $\gamma$  chain. This finding is rather surprising in view of

the extensive polymorphism which was shown to be expressed predominantly on the light chain  $\beta$ , whereas variability of the  $\alpha$  chain was much more restricted [1]. A substantial homology relationship is also seen when the fragmentation patterns of the heavy and light chains are compared. Because by N-terminal radiosequence analysis only a rather sparse N-terminal homology was observed between I-A<sup>k</sup> $\alpha$  and I-A<sup>k</sup> $\beta$  polypeptides [11], one would expect that, like in the human system, the degree of homology is increasing when internal regions of both molecules are compared.

During alkylation studies on intact spleen cells we observed that the subunits  $\alpha$ ,  $\gamma$ ,  $\beta_1$ ,  $\beta_2$  each carry at least one free sulphhydryl group [5]. One could exploit the SH-target by attaching a radiolabeled alkylation agent and assessing the positional properties of the label by tryptic peptide mapping. Therefore, we extracted the individual polypeptide chains from a set of excised gel pieces (fig. 1), blocked the free SH-group with iodo [<sup>14</sup>C]acetic acid and subjected the proteins to tryptic peptide map analysis after complete reduction with DDT and alkylation with iodoacetamide. (See peptide maps in fig. 3).

Again  $\beta_1$  and  $\beta_2$  are highly homologous, confirming our assignment. The distribution of the strongly labeled <sup>14</sup>C-spots in the 4 peptide maps of  $\alpha$ ,  $\beta_1$ ,  $\beta_2$  and  $\gamma$  is also similar, indicating that the stretches of amino acids carrying the free sulphhydryl group comprise a conserved region in the heavy and light chains as well as in the  $\gamma$  chain.

This finding is consistent with data in [12]: From sequencing studies of genomic and cDNA clones of A<sup>k</sup> $\alpha$  and E<sup>k</sup> $\alpha$  position 195 of both heavy chains was indicated as occupied by a presumably free cysteine residue located in the transmembrane region. Furthermore, a comparison of the amino acid sequences of A<sup>k</sup> $\alpha$  and E<sup>k</sup> $\alpha$  revealed that the greatest similarity resides in the free cysteine-containing transmembrane region where 18 out of 23 amino acids are identical. However, the number of peptides generated by trypsin digestion of the carboxymethylated subunits  $\alpha$ ,  $\gamma$ ,  $\beta_1$ ,  $\beta_2$  (fig. 3) is greater than expected from the reported sequences of A<sup>k</sup> $\alpha$  and E<sup>k</sup> $\alpha$  [12]. Incomplete digestion of the hydrophobic region of the polypeptide chains could account for this phenomenon.

We next performed 2D tryptic fingerprints from [<sup>35</sup>S]methionine-labeled  $\alpha$ ,  $\gamma$  and  $\beta$  chains, obtain-

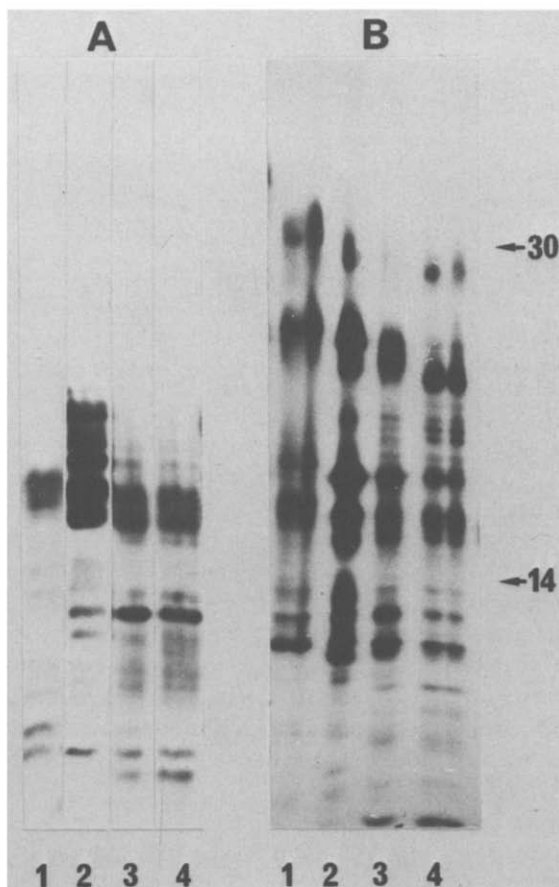


Fig. 2. Comparative SDS-PAGE profiles from [<sup>35</sup>S]methionine labeled I-A<sup>k</sup> subunits  $\alpha$  (1),  $\gamma$  (2),  $\beta_1$  (3) and  $\beta_2$  (4) isolated by preparative SDS-PAGE and digested with papain (A) and *Staph. aureus* V8 protease (B). The positions of <sup>14</sup>C-labeled markers (carboanhydrase and lysozyme) in kDa are indicated on the right.

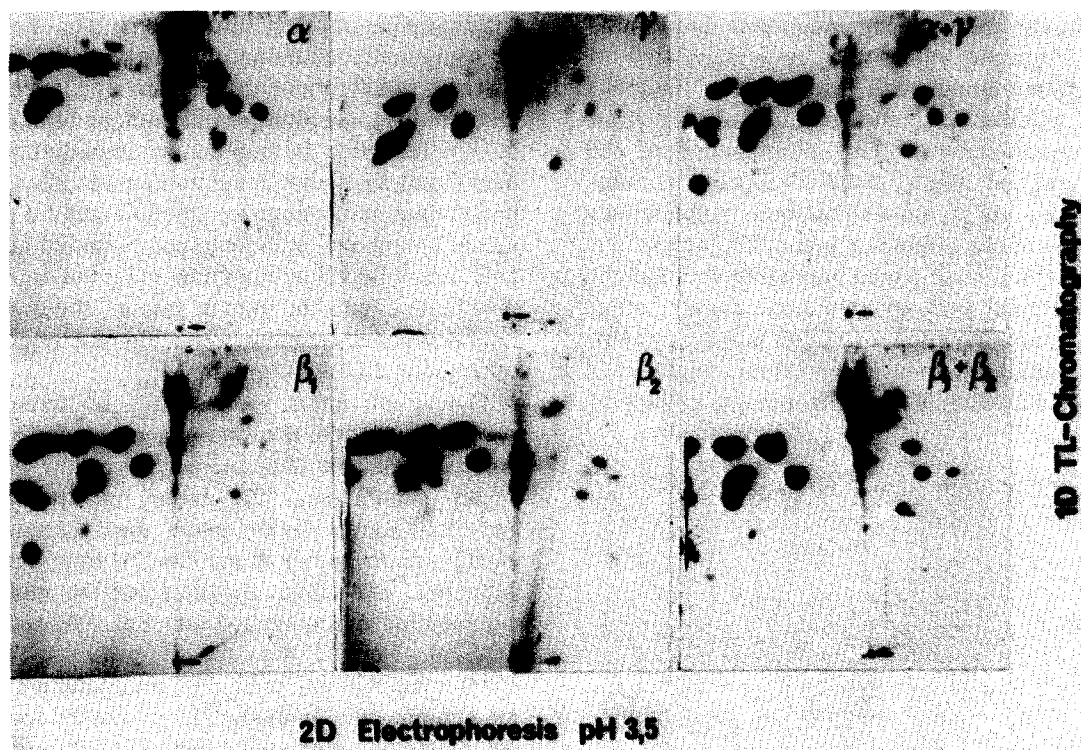


Fig. 3. Comparative two-dimensional maps of tryptic peptides from I-A<sup>k</sup> subunits  $\alpha$ ,  $\gamma$ ,  $\beta_1$  and  $\beta_2$  isolated by preparative SDS-PAGE in the absence of reducing agents and alkylated with iodo [<sup>14</sup>C]acetic acid (anode to the left; origin).

ed by an alternative purification procedure. I-A<sup>k</sup> specific components were recovered from a [<sup>35</sup>S]methionine labeled spleen cell NP 40 lysate by

passage over a monoclonal immunoadsorbent (10-2.16 bound to Sepharose CL 4B) as in [6]. The pooled class II antigen fractions were concentrated by reduced pressure dialysis and subjected to 2D O'Farrell gel electrophoresis with subsequent electroblotting onto nitrocellulose sheets. A test aliquot of the combined fractions was applied to an analytical O'Farrell gel to reveal the complexity of the I-A<sup>k</sup> isolate (fig. 4A).  $\alpha$ ,  $\gamma$  and  $\beta$  spots were cut from the nitrocellulose according to the corresponding autoradiogram (fig. 4B) and the

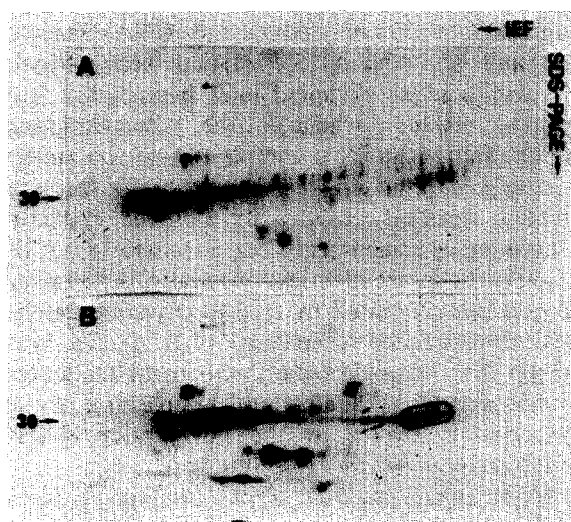


Fig. 4. 2D O'Farrell gel pattern of I-A<sup>k</sup> molecules ( $5 \times 10^4$  cpm) immunoprecipitated by mcab 10-2.16 (anti-I-A<sup>k</sup>) from [<sup>35</sup>S]methionine-labeled C3H NP40 spleen cell extract: (A) acidic end of the gel is on the right; (B) electroblot of I-A<sup>k</sup> molecules ( $2 \times 10^6$  cpm) separated on a 2D O'Farrell gel and transferred to nitrocellulose. Radioactive spots corresponding to  $\alpha$ ,  $\gamma$  and  $\beta$  (encircled) were isolated from the cellulose sheet using the autoradiogram as template.

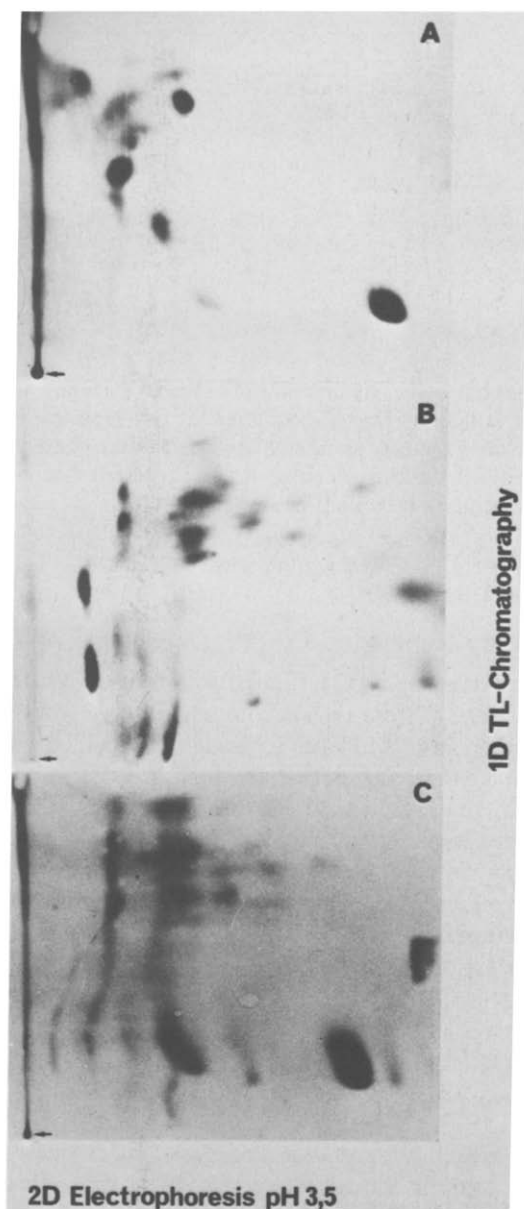


Fig. 5. Comparative two-dimensional maps of [ $^{35}\text{S}$ ]methionine labeled tryptic peptides from  $\alpha$  (A),  $\gamma$  (B) and  $\beta$  (C) subunits isolated by 2D gel electrophoresis and electroblotting (anode on the left; origin).

proteins were reduced, alkylated and digested with TPCK-trypsin. Released peptides were separated on thin-layer cellulose plates as above.  $\beta$  and  $\gamma$  shared 59% of their [ $^{35}\text{S}$ ]methionine peptides (fig. 5). Similarly,  $\alpha$  and  $\gamma$  were about 46% homologous. Thus the combined findings provide

convincing evidence that

- (i) The 3 major subunits of the I-A<sup>k</sup> specific molecule are structurally related;
- (ii) The degree of homology is increasing towards the carboxy-terminal end of the polypeptide chains;
- (iii) According to our test systems the invariant  $\gamma$  chain and the highly polymorphic  $\beta$  chain have more portions of their sequence in common than  $\gamma$  and  $\alpha$ .

Thus  $\gamma$ , as  $\alpha$  and  $\beta$ , could possess a domain-like structural organization. The 3 chains are probably descendents from a primordial protein.

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