Mouse invariant chain γ exhibits structural homology to both polymorphic subunits of the α,β-core complex of I-A^k antigens

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The 3 major constituents of the I-A^k subregion-associated complex α, β and γ were obtained from splenocytes in homogeneous form by differential isolation methods. α, β and γ 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 γ exhibits extensive structural homology to the polymorphic β-light and the α-heavy chain. Thus, although not being encoded within the MHC γ 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 Aα (35 kDa) and Aβ (28 kDa) whereas the I-E complex is composed of Eα (36 kDa), mapping to the I-E region and Eβ (31 kDA) mapping to the I-A region (review [1]). Intracellularly both α, β core complexes are found associated non-covalently with the invariant chain Ii (γ) (32 kDA), which is a membrane glycoprotein as ar5e the heavy and light chains [2]. The γ chain is not encoded within the MHC [3,4].

Recently we reported that the light chain β of the I-A^k complex can be separated on one-dimensional (1D) SDS–polyacrylamide gels in the absence of reducing agents into two distinct molecular species: β_1 (27 kDa) and β_2 (25 kDa) [5]. The structural comparisons presented here support our notion that β_1 and β_2 are two conformational variants of the light chain β. Surprising structural similarities are apparent between the invariant chain γ and the polymorphic β chain and to a lesser extent between γ and the α chain, suggesting that the γ chain is structurally related to the two MHC encoded class II proteins α and β.

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

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 μCi . 10^7 cells^{-1} ml^{-1} [^{35}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^k) [7] and adsorption to protein A–Sepharose (Pharmacia) followed by preparative 1D SDS–PAGE on long (12 × 23 cm) polyacrylamide gradient gels was as outlined

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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 α, γ, β_1 and β_2 was done by reacting portions $\sim 10^4$ cpm ^{35}S of the subunits with iodo-[^{14}C]acetic acid (12 μCi) (Amersham, Braunschweig) in 100 μl 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 2×10^5 cpm of the ^{14}C-labeled protein in 100 μl 0.1 M NH_4HCO_3 (pH 8.0) with 2 μl of 1% TPCK-trypsin in H_2O for 1 h at 37°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 × 20 cm) (Polygram Cel 300, Machery-Nagel, Düren) applying ascending chromatography in H_2O/*n*-butanol/pyridine/acetic acid 24:30:20:6 (by vol.) for the first dimension and electrophoresis in H_2O/pyridine/acetic acid 756:4:40 (by vol.) (600 V at 10°C/90 min) for the second dimension. The dried plates were placed on Kodak XR-5 film for autoradiography. Alternatively, highly purified [^{35}S]methionine-labeled subunits α, γ and β were prepared by immunoaffinity chromatography of [^{35}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.

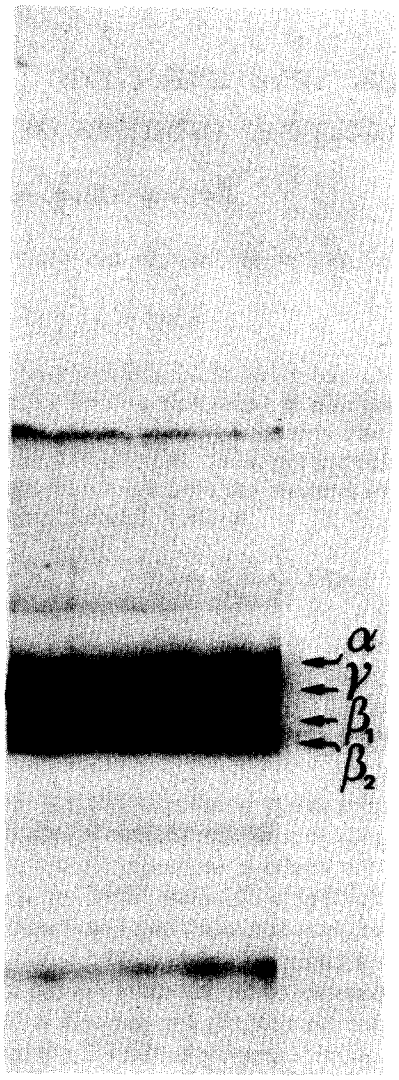

Fig. 1. Preparative SDS–PAGE separation of [^{35}S]methionine labeled I-A^k antigens in the absence of reducing agents on a linear 5–20% polyacrylamide gradient gel.

3. RESULTS AND DISCUSSION

I-A^k molecules were isolated from [^{35}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 α, invariant chain γ and two conformational variants of the light chain β, designated β_1, and β_2 [5]. The high resolution of this preparative run permitted the isolation of homogeneous polypeptides from the wet gel using the autoradiogram as template. Electrophoresis of test aliquots of isolated α, γ, β_1 and β_2 polypeptides on 2D O'Farrell gels indicated that homogeneity of all four I-A^k subunits had been achieved (not shown). To elucidate the structural relationship of α, γ, β_1 and β_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 β_1 and β_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 α polypeptide shows striking homology with the β chains and, to a lesser extent, with the α chain. This finding is rather surprising in view of the extensive polymorphism which was shown to be expressed predominantly on the light chain β, whereas variability of the α 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^k α and I-A^k β 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.

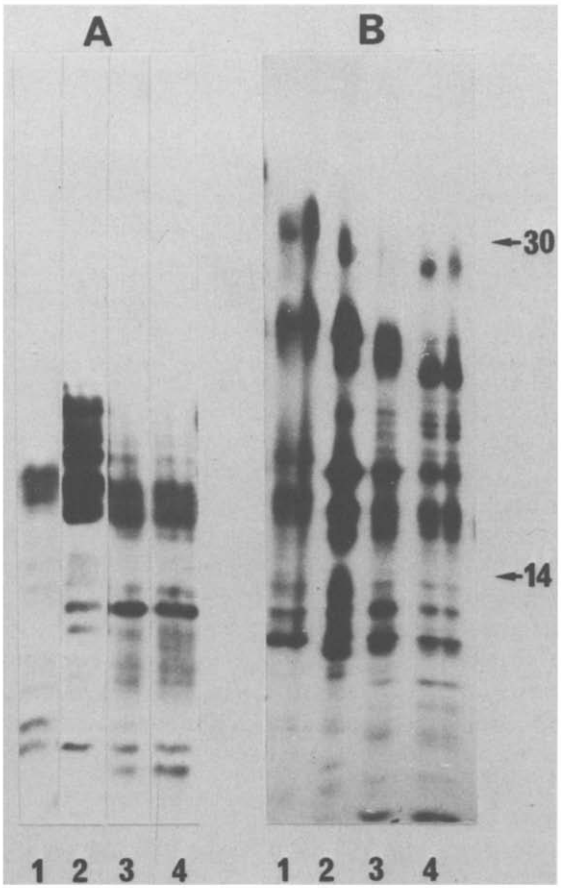

Fig. 2. Comparative SDS–PAGE profiles from [^{35}S]methionine labeled I-A^k subunits α (1), γ (2), β_1 (3) and β_2 (4) isolated by preparative SDS–PAGE and digested with papain (A) and *Staph. aureus* V8 protease (B). The positions of ^{14}C-labeled markers (carboanhydrase and lysozyme) in kDa are indicated on the right.

During alkylation studies on intact spleen cells we observed that the subunits α, γ, β_1, β_2 each carry at least one free sulphydryl 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 [^{14}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 β_1 and β_2 are highly homologous, confirming our assignment. The distribution of the strongly labeled ^{14}C-spots in the 4 peptide maps of α, β_1, β_2 and γ is also similar, indicating that the stretches of amino acids carrying the free sulphydryl group comprise a conserved region in the heavy and light chains as well as in the γ chain.

This finding is consistent with data in [12]: From sequencing studies of genomic and cDNA clones of $A^k\alpha$ and $E^k\alpha$ 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 $A^k\alpha$ and $E^k\alpha$ 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 α, γ, β_1, β_2 (fig. 3) is greater than expected from the reported sequences of $A^k\alpha$ and $E^k\alpha$ [12]. Incomplete digestion of the hydrophobic region of the polypeptide chains could account for this phenomenon.

We next performed 2D tryptic fingerprints from [^{35}S]methionine-labeled α, γ and β chains, obtain-

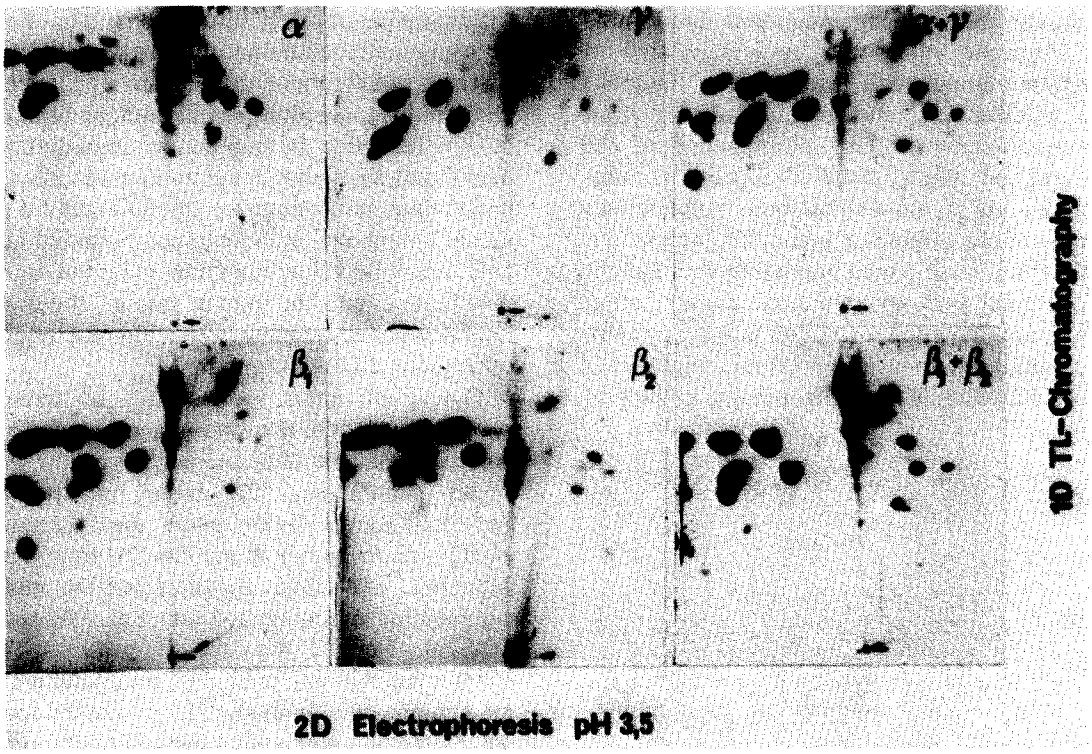

Fig. 3. Comparative two-dimensional maps of tryptic peptides from I-A^k subunits α, γ, β_1 and β_2 isolated by preparative SDS–PAGE in the absence of reducing agents and alkylated with iodo [^{14}C]acetic acid (anode to the left; origin).

ed by an alternative purification procedure. I-A^k specific components were recovered from a [^{35}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^k isolate (fig. 4A). α, γ and β spots were cut from the nitrocellulose according to the corresponding autoradiogram (fig. 4B) and the

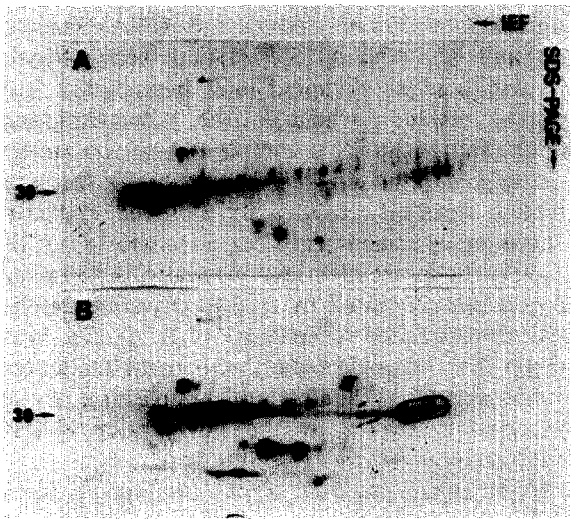

Fig. 4. 2D O'Farrell gel pattern of I-A^k molecules (5×10^4 cpm) immunoprecipitated by mcab 10-2.16 (anti-I-A^k) from [^{35}S]methionine-labeled C3H NP40 spleen cell extract: (A) acidic end of the gel is on the right; (B) electroblot of I-A^k molecules (2×10^6 cpm) separated on a 2D O'Farrell gel and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

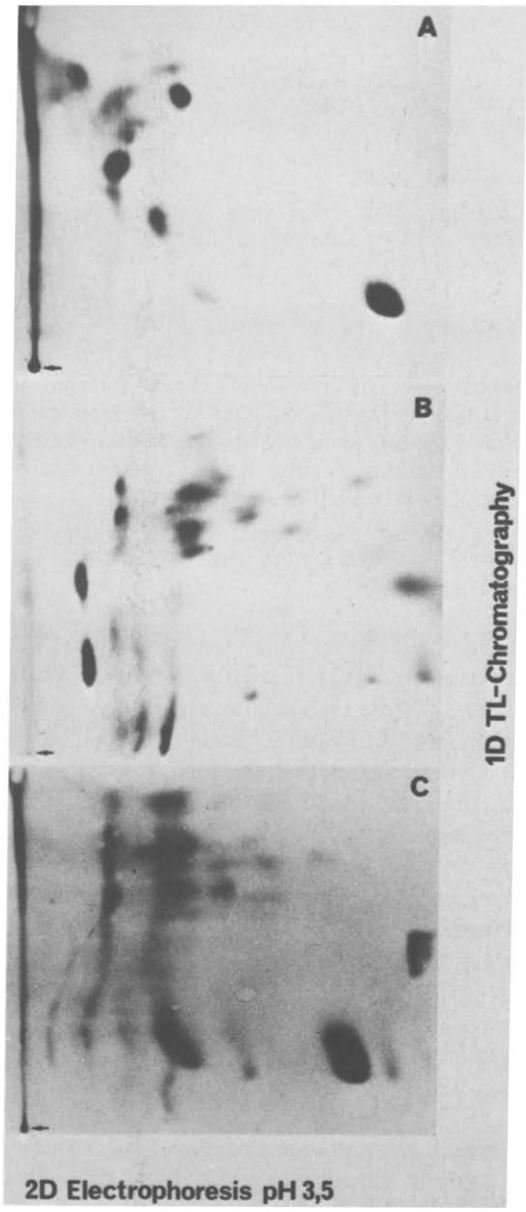

Fig. 5. Comparative two-dimensional maps of [^{35}S]methionine labeled tryptic peptides from α (A), γ (B) and β (C) subunits isolated by 2D gel electrophoresis and electroblotting (anode on the left; origin).

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

(i) The 3 major subunits of the I-A^k 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 γ chain and the highly polymorphic β chain have more portions of their sequence in common than γ and α.

Thus γ, as α and β, could possess a domain-like structural organization. The 3 chains are probably descendents from a primordial protein.

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