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A Homozygous Human Cell Line Contains Three Subsets of HLA-DR-like Antigens Distinguishable by Amino Acid Sequencing†

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ABSTRACT: The major histocompatibility complex (HLA in humans) contains a relatively large set of gene loci in the HLA-D/DR region responsible for regulation of the immune response. The structural dissection of the protein products of these loci is a necessary accompaniment to understanding of this response. In this study, two subsets of HLA-DR-like molecules have been separated by using monoclonal antibodies, and their component α and β chains have been subjected to amino acid terminal sequencing. The results from this sequencing experiment show three differences in the first 14 residues of the β chains and no differences in the first 15 residues of the α chains. These data along with previous sequencing of the DC-1 antigen [Bono, M. R., & Strominger, J. L. (1982) *Nature (London) 299*, 836–838] demonstrate that three distinct subsets of HLA-DR-like antigens are expressed by a homozygous human lymphoblastoid cell line.

Initiation, intensity, and specificity of the immune response to extrinsic and intrinsic antigens are controlled by the products of genes in the major histocompatibility complex (MHC)[1] (Strominger et al., 1981; Benacerraf, 1981). This large set of genes was originally defined in mice by the rejection of grafts or the proliferation of lymphocytes between individuals. The loci in the MHC were divided into three classes on the basis of either structure or function of their products, or a combination of both (Klein et al., 1981). The class I genes encode a polypeptide chain of 44 000 daltons, which forms a heterodimer with β_2-microglobulin (12 000 daltons). This functional unit is distributed on virtually all nucleated cells in vertebrates. These genes are the H-2K, D, and L loci in the mouse and the HLA-A, B, and C loci in humans. The class II genes encode polypeptide chains of two different sizes (α and β chains). The regions that contain these loci are called HLA-D/DR in humans and Ia in mice. The glycoproteins encoded by the these loci have a restricted cellular distribution, indicative of their role in lymphocyte interactions. The apparent molecular weight of these chains varies from species to species and even within species. In humans, the HLA-DR glycoproteins are 34 000 (α chains) and 29 000 (β chains) daltons, but nevertheless they are remarkably similar to the class I antigens in structure. Class III genes encode complement components, whose functional and structural connection with class I and II gene products is problematic, although they also function in the immune system.

Serological studies have revealed 10 alleles in the HLA-D region, defined by cellular reactivity, and 12 in the HLA-DR region, defined by alloantisera (Terasaki, 1980). However, subdivision of the HLA-D/DR region has not been accomplished to the extent that it has in the murine system, where I-A, I-J, and I-E subregions are relatively well-defined and subregions I-B and I-C have been suggested. Only two products of the Ia region (encoded by four genes in the I-A and I-E subregions) have been identified, each with polypeptides of about 28 000 and 33 000 daltons. Amino-terminal sequence data have revealed homology between DR and I-E polypeptides (McMillan et al., 1977; Allison et al., 1978) and between the closely related specificity DC-1 and I-A polypeptides [Bono & Strominger, 1982; see also Goyert et al. (1982)]. Herein we report the amino-terminal sequences of two antigenically distinguishable subsets of HLA-DR antigens from an HLA-DRw6 homozygous cell line separated by using monoclonal antibodies. The β chains of these molecules show three differences in the first 14 residues, while their α chains are identical for the first 15 residues. Thus, there are at least three sets of HLA-DR-like antigens expressed in a homozygous

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[1] Abbreviations: MHC, major histocompatibility complex; NaDodSO$_4$, sodium dodecyl sulfate; DOC, deoxycholate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride.

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human cell line [the DC-1 subset studied previously (Tosi et al., 1978; Shackelford et al., 1981b; Bono & Strominger, 1982) and the two DR subsets] encoded by three β-chain genes and at least two α-chain genes.

Materials and Methods

Isolation of DR Antigens. Human DR antigens were prepared from a homozygous B lymphoblastoid cell, LB (DRw 6,6). Solutions of the membrane proteins were generated by incubation of cells for 30 min at 4 °C in a buffer consisting of 2% NP-40, 0.01 M Tris (pH 7.8), 0.14 M NaCl, 1 mM $MgCl_2$, and 0.1 mM PMSF. The ratio of cells to buffer solution was 1 g of cells/4 mL of solution. After incubation, the mixture was centrifuged at 150000*g* for 1 h, and the supernatant was recovered and centrifuged for an additional 20 min at 12000*g*. For removal of actin, the resulting lysate was then passed over a column of Sepharose 4B-CL to which normal rabbit serum had been coupled. The lysate was then passed over a column of the monoclonal antibody LKT111 (Bono et al., 1979) immobilized on Affi-gel 10 (Bio-Rad). The eluate from this column was recycled over the LKT111 column for 24 h at a flow rate of no greater than 5 mL/h. The eluate was then cycled over a column of the monoclonal antibody L243 (Lampson & Levy, 1980) under the same conditions. The columns were then washed with no more than 5 column volumes of a buffer containing 0.14 M NaCl, 0.1% DOC, and 0.01 M Tris, pH 8.0. Bound proteins were then eluted from the columns by washing them with a buffer containing 0.5% DOC, 5% glycerol, and 0.1 M Tris, pH 11.5. Fractions were collected and neutralized with 1 N HCl, and aliquots were taken and treated with a 5-fold excess (v/v) of acetone to precipitate protein. The resulting precipitates were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis to locate the DR molecules (Laemmli, 1970). Fractions containing the DR polypeptides were dialyzed against 0.1% $NaDodSO_4$ and lyophilized.

Separation of α and β Chains. All operations and chromatography were carried out at 37 °C. The lyophilized DR molecule was dissolved in a buffer containing 0.1 M sodium phosphate, 0.1% $NaDodSO_4$, and 1 mM DTT (pH 6.4) and applied to a column of hydroxylapatite (Bio-Rad) previously equilibrated with the same buffer. After application of the sample to the column, a linear gradient elution was begun, with the elution buffer being 0.5 M sodium phosphate, 0.1% $NaDodSO_4$, and 1 mM DTT (pH 6.4). This procedure resulted in a separation of α and β chains, the β chain eluting at lower ionic strength (Freed, 1980). Fractions were collected and aliquots taken, diluted with Laemmli (1970) sample buffer, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. Fractions containing the respective polypeptide chains were dialyzed against 0.1% $NaDodSO_4$ and lyophilized.

Amino Acid Sequence. Polypeptides were sequenced in a Beckman 890C Sequenator, modified with a cold trap. Samples were loaded in 0.1% $NaDodSO_4$, along with 3 mg of Polybrene (Klapper et al., 1978). The resulting phenylthiocarbamyl derivatives were converted to their more stable phenylthiohydantoins (PTHs) and analyzed by high-pressure liquid chromatography on a Hewlett-Packard 1084B instrument with a 10-min program (W. Lane, unpublished results).

Results and Discussion

In a previous study (Bono & Strominger, 1982), we have separated the DR subset of antigens in a DRw6 homozygous cell line (LB) from the DC subset. The DR subset was clearly complex, as evidenced by the presence of at least two β chains in the preparation. The same complexity has been observed

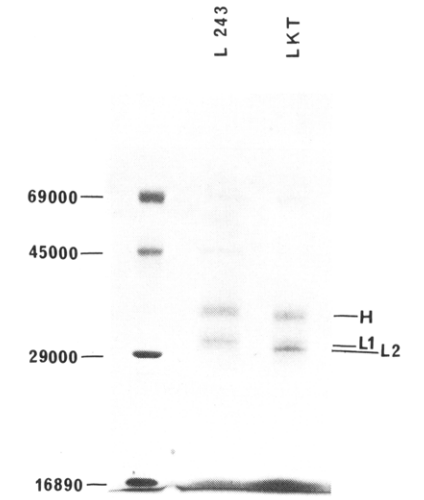

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of isolated DR molecules eluted from LKT111 and L243 monoclonal antibody columns. Gels were 10% acrylamide.

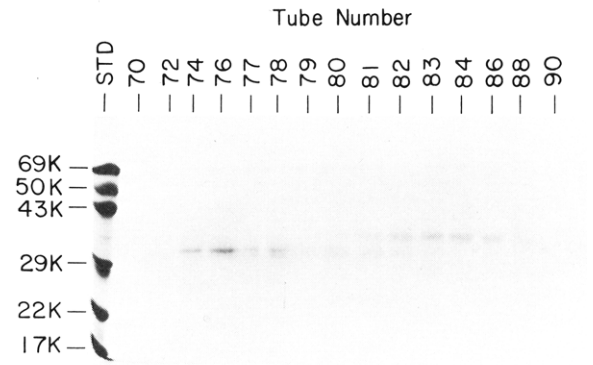

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of fractions eluted from a hydroxylapatite column during gradient elution. The column was run and samples were treated as described under Materials and Methods.

in all other homozygous DRw6 cell lines examined, including Arent, WT46, WT52, and HKK (Shackelford et al., 1981a,b, 1982; Shackelford & Strominger, 1982; D. Shackelford, B. Eibl, and J. L. Strominger, unpublished results). The monoclonal antibody LKT111 (Bono et al., 1979) reacted preferentially with one of these subsets of human DR molecules; the other subset was preferentially recognized by the monoclonal antibody L227 (Lampson & Levy, 1980). The LKT111 subset is characterized by a β chain of slightly greater mobility on $NaDodSO_4$–polyacrylamide gel electrophoresis and differing isoelectric focusing pattern than the L227 subset. By use of the LKT111 antibody to remove one subset, followed by the L243 (Lampson & Levy, 1980) antibody, which recognizes both subsets, a separation has been effected. (The separation could also be affected by using LKT111 in concert with L227 monoclonal antibody. However, at the time these experiments were carried out, we were experiencing difficulty in producing L227. This difficulty has now been overcome.) This separation produced two subsets having β chains of differing mobilities (Figure 1). The chains of each subset were separated by chromatography on hydroxylapatite (Freed, 1980) (Figure 2) and then subjected to analysis.

The amino-terminal sequence data clearly demonstrate three differences between the two β-chain subsets in the first 14 residues (Figure 3). Thus, these results suggest that these chains are encoded by different genes. The argument that the two β chains are separately encoded in the same haplotype rests

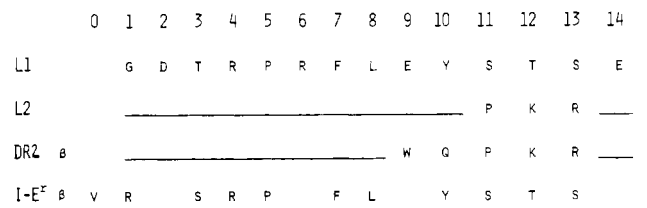

FIGURE 3: Amino acid sequence for DR and I-region β chains. L1 is the β chain isolated from the DR molecule eluted from the L243 column. L2 is the β chain isolated from the DR molecule eluted from the LKT111 column. The DR2 β-chain sequence is from Kratzin et al. (1981). The mouse E/C^r β-chain sequence is from Cook et al. (1979).

on the homozygosity of the LB cell line. The LB cell line has been typed as HLA-A 28,28, -B40,40, -Cw3,w3, and -DRw6,w6 and, in addition, the same complexity has been observed in four other DRw6 homozygous cell lines (vide supra). The α-chain amino-terminal sequences were identical for the first 15 residues and showed no differences with respect to the published sequence (Strominger et al., 1981). However, a slight difference in gel mobility in the two heavy chains may be apparent in Figure 1, and in any case much more extensive sequence information will be necessary to establish whether or not the heavy chain associated with the LKT111 subset of DR antigen and that associated with the L227 subset are identical.

Kratzin et al. (1981) have obtained the complete sequence of the two extracellular domains of a β chain from a DR2 antigen (residues 1–198). They also suggested the possibility that their preparation was heterogeneous at residues 60–69 and that several light chains might be present in the preparation. In fact, careful examination of the L1 and L2 patterns (Figure 1) suggests that each subset could consist of a family of closely related molecules. Comparison of the amino-terminal sequence of the DR2 β chain with the two amino-terminal sequences of the L1 and L2 light chains shown here reveals a high conservation of sequence homology with L2 except in residues 9 and 10 and with L1 except in residues 9–13. Similarly, the homology to the mouse I-E β chain is striking, as has been previously noted, but particularly interesting in the present context is the homology between L1 and the murine $I\text{-}E^r$ β sequence (Cook et al., 1979), especially at residues 10–13. A number of investigators have observed an apparent multiplicity of α- and β-chain species in two-dimensional gels, employing immunoprecipitation of material from radioactively labeled cells (Markert & Cresswell, 1980, 1982a,b; Shackelford & Strominger, 1980, 1982; Shackelford et al., 1981a,b, 1982; deKrester et al., 1982; Karr et al., 1982). Such a multiplicity of protein products could be either the result of artifacts of isolation [see discussion in Shackelford & Strominger (1980)], or posttranslational modifications, or they could represent the products of separate genes. Isolation and amino acid sequencing and mutational studies are both methods of distinguishing these possibilities. The amino acid sequence data presented in this paper provide the first definitive evidence that more than one DR β chain is expressed in a homozygous human cell and that there are at least two subsets of DR antigens expressed in addition to the DC-1 subset.

Immunogenetic data have revealed an increasing complexity of the DR region in recent years. In addition to the originally defined HLA-D determinant (defined by mixed lymphocyte reactivity) and HLA-DR determinants defined by allospecific antibodies, a number of alloantisera have defined "supertypic" specificities referred to as DC1 (MB1, MT1, LB12), MB2, MB3, and MT1–MT6 (Terasaki, 1980). Evidence is accumulating that some, if not all, of these serological reactivities are due to molecules of polypeptide composition similar to but distinct from the DR antigens. Furthermore, an additional locus called SB (secondary B cell locus), which provides stimulation in mixed lymphocyte reaction, has been described and apparently also encodes one of the chains of a similar molecule (Shaw et al., 1982). The relationship among these various specificities is not presently clear. In this and a previous study (Bono & Strominger, 1982), however, we have defined the three major subsets of DR-like antigens present on a homozygous HLA-DRw6 human cell line. In these three subsets, all three of the light chains (Shackelford et al., 1982; D. Shackelford and B. Eibl, unpublished results) and at least the DC-1 heavy chain (C. Auffray, M. Roux-Dosseto, J. Lillie, A. Ben Nun, J. Seidman, and J. L. Strominger, unpublished results) must be polymorphic. The exact correspondence of these subsets and of the polymorphisms that they represent with reactivities defined by allospecific antisera and by cellular reactivity remains to be accomplished. It seems likely, however, that the predominant alloantisera define a β-chain polymorphism of one of the DR subsets (Shackelford et al., 1982) and that the MT antisera define the heavy-chain polymorphism of the DC-1 subset (C. Auffray et al., unpublished results).

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Articles

Rat Liver Thioredoxin and Thioredoxin Reductase: Purification and Characterization†

Mikaela Luthman and Arne Holmgren*

ABSTRACT: A reproducible scheme has been developed for the preparation of rat liver thioredoxin and thioredoxin reductase (EC 1.6.4.5) by using assays based on reduction of insulin and 5,5′-dithiobis(2-nitrobenzoic acid), respectively. Both proteins were purified to homogeneity, as judged from polyacrylamide gel electrophoresis. Thioredoxin had a molecular weight of 12000 and contained about 110 amino acids including 4 half-cystines and an NH_2-terminal valine. Peptide maps of reduced and carboxymethylated thioredoxin showed that the protein had the active center sequence -Cys-Gly-Pro-Cys-Lys-Met- characteristic of thioredoxins also from procaryotes. Prolonged air oxidation of fully reduced thioredoxin created inactive, aggregated disulfide-containing molecules. Thioredoxin reductase showed a subunit molecular weight of 58000 and a native molecular weight of 116000. The enzyme was highly specific for NADPH with a K_m of 6 μM. It contained FAD as prosthetic group and was sensitive to inhibition by arsenite. Thioredoxin reductase had a K_m of 2.5 μM for rat and calf liver thioredoxin and a K_{cat} of 3000 min^{-1}.

The thioredoxin system, thioredoxin, thioredoxin reductase, and NADPH, is a widespread thiol-dependent electron transport system [reviews by Williams (1976) and Holmgren (1980, 1981)]. Thioredoxin was originally purified from *Escherichia coli* as a dithiol hydrogen donor for the enzyme ribonucleotide reductase (Thelander & Reichard, 1979) and for enzymes catalyzing the reduction of sulfoxides and sulfate [see Holmgren (1980, 1981)]. In addition, the thioredoxin system promotes the reduction of certain protein disulfides by NADPH through a combination of reactions 1 and 2.

$$\text{thioredoxin-}S_2 + \text{NADPH} + H^+ \xrightarrow{\text{thioredoxin reductase}} \text{thioredoxin-}(SH)_2 + \text{NADP} \quad (1)$$

$$\text{thioredoxin-}(SH)_2 + \text{protein-}S_2 \rightarrow \text{thioredoxin-}S_2 + \text{protein-}(SH)_2 \quad (2)$$

E. coli thioredoxin-S_2 (M_r 11700) has been extensively characterized (Homgren, 1981). It contains an oxidation-reduction active cystine disulfide (-Cys_{32}-Gly-Pro-Cys_{35}-) in a unique protrusion of the three-dimensional structure (Holmgren et al., 1975). Thioredoxin reductase (EC 1.6.4.5) from *E. coli* (M_r 70000) is a dimer of two apparently identical subunits, each containing FAD and an oxidation-reduction active disulfide (Williams, 1976).

Mammalian thioredoxins and thioredoxin reductase have so far remained largely uncharacterized. The lack of readily available standardized assay systems and the inactivation and apparent aggregation of the proteins in the absence of thiols have complicated studies (Holmgren, 1980). Homogeneous preparations of thioredoxin have been obtained from the *Novikoff* ascites rat tumor (Herrmann & Moore, 1973), by using a partially purified tumor ribonucleotide reductase, and from calf liver (Engström et al., 1974), by an assay utilizing the reduction of insulin disulfide by NADPH in the presence of a partially purified calf thioredoxin reductase. It is not known if *Novikoff* tumor thioredoxin is identical with the rat liver thioredoxin, which has beforehand only been partially purified (Larson & Larsson, 1972).

Thioredoxin reductase has been purified to about 95% homogeneity from the Novikoff ascites rat tumor (Chen et al., 1977) and also partly purified from rat liver (Larsson, 1973) and calf liver and thymus (Holmgren, 1977). The tumor enzyme was reported to have an isoelectric point of 5.1 by isoelectric focusing (Chen et al., 1978). Variant forms with isoelectric points of 4.9 and 4.7 were observed in adult rat liver extracts, but the implications of these findings remain unclear

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